Universidade de Aveiro
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Departamento de Química

Mafalda Sofia Mendes Gonçalves

Nº Mec.: 59000

EFEITO DA PASTEURIZAÇÃO E DA ALTA PRESSÃO EM LEITE E COLOSTRO DE BURRA

EFFECT OF PASTEURIZATION AND HIGH PRESSURE IN DONKEY MILK AND COLOSTRUM

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia, no Ramo de Biotecnologia Industrial e Ambiental, realizada sob a orientação científica do Doutor Jorge Manuel Alexandre Saraiva, Investigador Auxiliar do Departamento de Química da Universidade de Aveiro.

Dedico este trabalho aos meus pais e à minha irmã.

Júri

Presidente

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Palavras-chave Leite de burra, colostro, pasteurização, alta pressão, lisozima, imunoglobulinas, microorganismos

Resumo

Quando a amamentação não é possível ou após o desmame, encontrar uma alternativa nutricional adequada torna-se imprescindível. Fórmulas comerciais infantis têm sido desenvolvidas como substitutos nutricionais para imitarem o leite materno. Actualmente, estudos clínicos demonstraram que o leite de burra poderia substituir a amamentação de recém-nascidos afectados por graves alergias mediadas pela imunoglobulina E (IgE) do leite de vaca, cuja composição é muito semelhante ao leite materno. A alta pressão (AP) é uma tecnologia não-térmica capaz de fornecer alimentos seguros com características semelhantes aos alimentos não processados, garantindo a inativação microbiana e mantendo as propriedades nutricionais e funcionais.

Este trabalho teve como objectivo primordial avaliar e comparar o efeito da pasteurização térmica e da AP (400, 550 e 625 MPa durante 2,5, 10 e 30 min a 8 °C) na qualidade microbiana, na concentração das imunoglobulinas A, M e G (IgA, IgM e IgG), e na actividade da enzima lisozima do leite e colostro de burra.

Os resultados microbiológicos revelaram que os valores de log UFC/mL em leite não processado estavam acima dos valores máximos permitidos no 6º dia de armazenamento, enquanto que após tratamento térmico, os valores para os microrganismos totais, *Enterobacteriaceae* e coliformes foram 4.21, 3.0 e 4.04 log UFC/mL ao dia 30 de armazenamento, respectivamente. Entretanto, no colostro não processado foram apenas detetados microrganismos totais e *Enterobacteriaceae,* com os valores máximos permitidos nos dias 4 e 7, respectivamente. AP (400 e 550 MPa durante 10 min) inativou todos os microrganismos estudados para valores abaixo dos níveis de quantificação pelo menos até ao dia 30 no leite e 40 no colostro.

Relativamente à actividade da lisozima observou-se que no leite manteve-se em todos os tratamentos de AP utilizados com tempos de pressurização menores (2.5 e 10 min), enquanto com 30 min se observou um decréscimo entre 30 e 40%. No caso do colostro observou-se uma maior redução a 625 MPa, 10 e 30 min (40%). A pasteurização, por outro lado, resultou em perdas significativas da actividade desta enzima, especialmente no colostro (40%).

Em relação ao conteúdo de IgA, tanto no leite como no colostro apenas se observou nas amostras não processadas. A 625 MPa (10 e 30 min) não se detectou IgM no colostro e IgG no leite. Sendo que no colostro, os valores de IgG foram superiores à amostra não processada a 550 (2.5 e 10 min, 208 e 143%) e 625 MPa (10 e 30 min, 412 e 260%).

Este trabalho demonstrou que tratamentos de alta pressão na pasteurização do leite e colostro (acima de 550 MPa/10 min) podem garantir a segurança microbiológica e preservar a actividade da lisozima e conteúdo da IgG, sendo uma boa alternativa à pasteurização térmica.

Keywords Donkey milk, colostrum, pasteurization, high pressure, lysozyme, immunoglobulins, microorganisms

Abstract

When breast-feeding is not possible or after weaning, finding an adequate alternative nourishment becomes mandatory. Commercial infant formulas have been developed as nutritional substitutes for breast milk. Actually, clinical studies have demonstrated that donkey milk could substitute breast feeding in infants affected by severe IgE-mediated cow milk allergies, whose composition is very similar to breast milk. High-pressure processing (HPP) is a non-thermal technology that can provide safe foods with similar characteristics to the unprocessed foods, causing microbial inactivation while maintaining its nutritional and functional properties.

The main goal of this work was to investigate and compare the effect of thermal pasteurization and HPP (400, 550 and 625 MPa for 2.5, 10 and 30 min at 8 °C) on microbial quality, in immunoglobulin A, M and G (IgA, IgM and IgG) concentrations and lysozyme activity in donkey milk and colostrum.

The microbiological results indicated that the log of CFU/mL of the raw milk were already above the maximum values allowed at the 6^{th} day of storage, while total aerobic mesophilic bacteria, *Enterobacteriaceae* and Coliforms for the thermal pasteurized milk were 4.21, 3.0 and 4.04 log CFU/mL at the 30^{th} day of storage, respectively. In raw colostrum total aerobic mesophilic bacteria and *Enterobacteriaceae*, were detected above the maximum values allowed at the 4^{th} and 7^{th} day, respectively. HPP (400 and 550 MPa at 8˚C, for 10 min) inactivated all the quantified microorganisms to values below the detection levels up to at least the 30^{th} day for milk and 40^{th} for colostrum.

Regarding the activity of lysozyme it was observed that in the milk its activity was kept in all AP treatments used for pressurization for 2.5 and 10 min. At 30 min occurred a decrease of about 30 to 40%. In the case of colostrum there was a greater reduction at 625 MPa, 10 and 30 min (40%). The thermal pasteurization, on the other hand, resulted in significant loss of the activity, especially in the colostrum (40%).

In relation to the content of IgA in both the colostrum and milk, it was observed only in samples not processed. At 625 MPa (10 and 30 min) IgM was not detected in colostrum and IgG not detected. In colostrum, IgG values were higher than the unprocessed sample at 550 (2.5 and 10 min, 208% and 143%) and at 625 MPa (10 and 30 min, 412% and 260%).

This work demonstrates that pressure treatments at the level necessary for milk pasteurization (up to 550 MPa/10 min) can guarantee microbial safety and preserve lysozyme activity and IgG content, being a good alternative to thermal pasteurization.

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AAF	Amino acids formulas
BSA	Bovine serum albumin
CMA	Cow's milk allergy
CMP	Cow's milk proteins
CMPA	Cow's milk protein allergy
D-value	Decimal reduction time
eHF	Extensively hydrolyzed formulas
FAO	Food and Agriculture Organization
HACCP	Hazard Analysis and Critical Control Points
HPP	High-pressure processing
IgA	Immunoglobulin A
IgD	Immunoglobulin D
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
Igs	Immunoglobulins
k	Reaction rate constant
LF	Lactoferrin
LPO	Lactoperoxidase
LTLT	Low-temperature long-time
SD	Standard deviation
SF	Soy formulas
WHO	The World Health Organization
β-Lg	β-Lactoglobulin
α-La	α-Lactalbumin

I. Introduction

The present study is divided into seven different sections (Section I). After this introductory section, the second consists in a comprehensive literature review and state of the art in what concerns composition and general characteristics of milk, human milk and its substitutes, donkey milk, bioactive proteins in donkey milk, the currently used thermal pasteurization method and the non-thermal high pressure processing (Section II). In section III, the objectives of this study will be mentioned. Section IV, the material and methods used during this work are described. Section V consists in the results obtained and the respective discussion, correlating with the available literature. Section VI features the global conclusions of the previous sections. Finally, section VII consists of one work to be done in this research area.

II. Literature Review

1. Historical Contextualization

Dairy products have been a part of human diet for more than 7,000 years and are generally defined as food produced from the milk of mammals (Piccione *et al.*, 2008). The history of milk beings in the Neolithic Age, a time when humans started the transition from hunting and gathering to a more settled way of life. This, in turn, allowed for new possibilities of adapting resources to acquire food. The most important, together with agricultural development, was the domestication of animals, which meant constant access to their meat, fur, and of course milk. The first attempts to domesticate ruminants (goats, cow, and sheep) began 11,000 years ago in the Middle East. Throughout the centuries, milk became a desirable and valuable food source wherever livestock animals were bred (Barłowska *et al.*, 2011; Huppertz, Fox*, et al.*, 2006).

Cattle are the most significant species in dairy production. Presently, the number of animals bred for dairy purposes is numerous, and in different regions around the world people have adapted their particular species to produce milk. In many regions buffalo milk is often used. Camel milk is also consumed in various countries that rear camel, such as Kenya, Somalia, Ethiopia, and Pakistan. Latin America has a wide variety of ruminants to choose from including camelids and llamas. Moose milk is popular in Russia and Sweden, whereas in Mongolia mare milk is commonly consumed and yak milk is used in Tibet. Sápmi habitants, in its turn, have been using reindeer milk for hundreds of years. The latest nutritional discovery is donkey milk, which is exceptionally similar to human milk in terms of protein composition. This similarity has made donkey milk very interesting for nutritionists, as it is thought to be less prone to cause allergy (Barłowska *et al.*, 2011).

Currently, global milk production is dominated by five animal species: dairy cattle, buffalo, goats, sheep, and camels. The major cow's milk producers worldwide are The European Union, The United States of America, India, and Russia. The production of buffalo milk is concentrated in two countries; nearly 92% of its worldwide production is in India and Pakistan. The largest producers of goat milk in the world are India and Bangladesh, and leaders among the European include Greece, Turkey, Romania, and Italy. Camel milk is almost exclusively produced in Somalia,

Ethiopia, Mali, Sudan, and Saudi Arabia (Barłowska *et al.*, 2011). Depending on the nature of animal ecology, evolution, genetics, and feeding management, milk composition and nutritional properties differ vastly among species (Nikkhah, 2011). The average intake of dairy products from livestock in many regions is much below the minimum daily requirements of about 70 g protein and 0.8 g calcium (Nikkhah, 2011, 2012). Cow's milk has been the major source of milk and dairy products in developed countries, especially in the Western world (Park, 2009).

1.1. Composition and general characteristics of milk

Milk is a fluid secreted by female mammals (Belitz *et al.*, 2009; Fox and Kelly, 2006), of which there are approximately 4.500 species, to meet the complete nutritional, and some of the physiological requirements of the neonate of the species (Fox, 2003). It is a good medium for the growth of many microorganisms (Nazzaro *et al.*, 2010; Šarić *et al.*, 2012; Varga, 2007; Zhang *et al.*, 2008), such as lactic acid bacteria, since it contains many nutrients and provides a suitable physical environmental for microbial growth (Nazzaro *et al.*, 2010; Zhang *et al.*, 2008). Its composition varies according to several factors, such as genetic, physiological, nature of animal ecology, nutritional, and environment conditions (Blasi *et al.*, 2008; Malacarne *et al.*, 2002; Nikkhah, 2011; Raynal-Ljutovac *et al.*, 2008).

Among the many valuable constituents in milk, the high levels of calcium play an important role in the development, strength, bones density in children and in the osteoporosis prevention in elderly people. Calcium has also been shown to be beneficial in reducing cholesterol absorption and in controlling body weight and blood pressure. Recent numerous research activities and advanced compositional identification of a large number of bioactive compounds in milk and dairy products have led to the discovery of specific biochemical, physiological, nutritional functionalities and characteristics that have strong potential for beneficial effects on human health. Four major areas of milk components bioactivity have been categorized: (1) gastrointestinal development, activity, and function; (2) infant development; (3) immunological development and function; and (4) microbial activity, including antibiotic and probiotic action (Park, 2009).

In addition to meeting the complete nutritional requirements of the neonate, milk serves several physiological functions, including protective (immunoglobulins and other antibacterial agents), digestive aids (enzymes and enzyme inhibitors, binding or carrier proteins) and growth factors/hormones (López Expósito and Recio, 2006; Paolo Polidori *et al.*, 2009). It contains several physiologically functional components including proteins, vitamins, as well as carotenoids and flavonoids with antioxidant properties (Simos *et al.*, 2011). The number, potency and importance of these agents are probably greater than previously thought and include direct-acting antimicrobial factors, anti-inflammatory substances and immunomodulators (López Expósito and Recio, 2006).

Consequently, milk is made of numerous bioactive compounds functioning beyond their nutritional importance, as these minimize the risks of cancers, traumas, and metabolic complexities (Nikkhah, 2012). As such, milk is considered the most natural functional biofluid (Fox and McSweeney, 1998; Nikkhah, 2012). These bioactives components include essential amino acids, casein and peptides, lactalbumins, immunoglobulins, nucleosides, unsaturated and conjugated linoleic acids, fat soluble vitamins, and calcium (Nikkhah, 2012). Figure 1 shows the major bioactive functional compounds derived from milk.

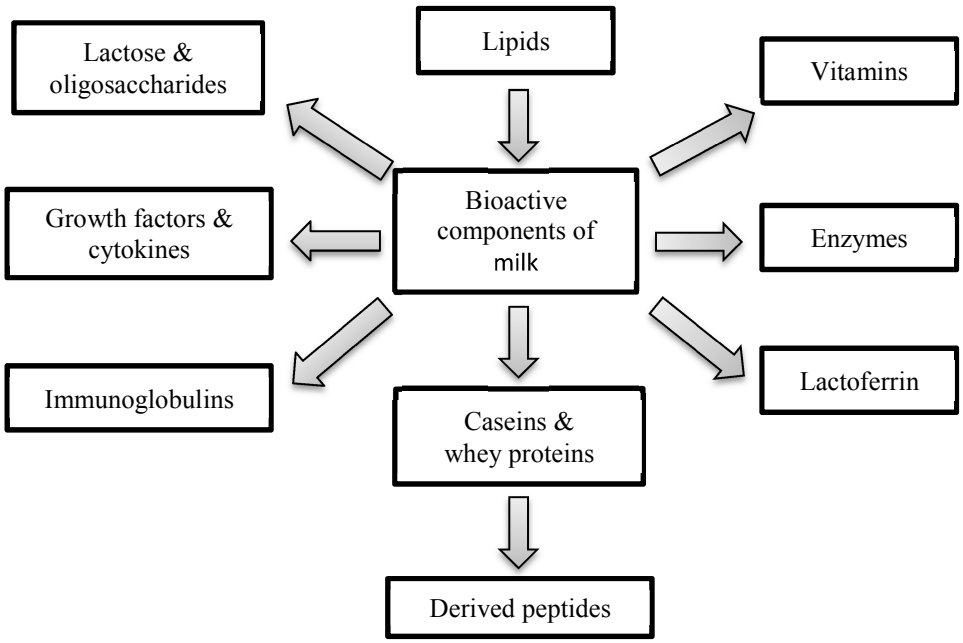

Figure 1. Schematic representation of major bioactive functional compounds derived from milk (Park, 2009).

1.2. Bioactive milk proteins

The main component of milk with a major impact on its nutritional value and technological suitability, is the protein (Barłowska *et al.*, 2011). Milk proteins are a heterogeneous group of compounds that differ in composition and properties (Barłowska *et al.*, 2011), and are probably its most important constituents, due to their unique properties, which are fundamental to the production and characteristics of dairy products, such as cheese or yogurt (Huppertz, Fox*, et al.*, 2006). These proteins, for ease description, could be classified into five main categories: caseins, whey proteins, milk fat globule proteins, enzymes, and other miscellaneous minor proteins (Fox and Kelly, 2006). However, milk proteins can also be divided into two classes, based on their solubility at pH 4.6: the soluble whey proteins and the insoluble caseins (Huppertz, Fox*, et al.*, 2006). Casein is the most important protein in milk (which represents ~80% of total milk protein), while the proportion of whey proteins is relatively low (~20% of total milk protein, consisting of β-lactoglobulin, α-lactalbumin, bovine serum albumin, and immunoglobulins) (Barłowska *et al.*, 2011; Huppertz, Fox*, et al.*, 2006; López-Fandiño, 2006; Park, 2009). Aside from nutritional values of milk, milkborne biologically active compounds such as casein and whey proteins have been found to be increasingly important for physiological and biochemical functions that have crucial impacts on human metabolism and health (Park, 2009).

Milk proteins appear to be an exciting link between nutrition, dietetics, and therapy. In fact, milk contains a variety of bioactive compounds with special properties associated with the development, growth, and survival of infants beyond those provided by nutrition alone (Zhang *et al.*, 2008). Immunoglobulins are among the first lines of protection that are delivered to the neonate through suckling and provide passively acquired immunity. Other proteins, such as the iron-binding protein, lactoferrin, and enzymes, including lysozyme and lactoperoxidase, play more of a direct role in inhibiting bacterial invasion (Clare *et al.*, 2003).

Immunoglobulins (Igs) act by a specific mode of action involving antigen-antibody reactions (Koenig *et al.*, 2005b). Mature milk contains 0.6-1.0 g Igs/L (~3% of total nitrogen) but colostrum contains up to 10% Igs, the level of which decreases rapidly post-partum (Fox, 2003). They are family of proteins with a range of protective bioactivities (Zagorska and Ciprovica, 2012). There are five classes, or isotypes, of Igs,

namely IgG, IgA, IgM, IgD and IgE, based on their physicochemical structures and biological activities (Walter L. Hurley and Theil, 2011). They are able to prevent the adhesion of microbes, inhibit bacterial metabolism, agglutinate bacteria, augment phagocytosis of bacteria, kill bacteria through activation of complement – mediated bacteriolytic reactions and neutralize toxins and viruses (Park, 2009). IgG always exists in monomeric form in human milk (Walter L. Hurley and Theil, 2011). They are comprised of two identical heavy chains and two identical light chains. On the other hand, IgA may occur in free form or complexed with the "J-chain" (representing the predominant Ig fraction, >90%) while IgM is a polymeric complex. Both IgA and IgM contain approximately 10% of carbohydrates. Synthesis of IgE is typically stimulated by response to allergic reactions and hypersensitivity (Clare *et al.*, 2003; Fox and Kelly, 2006). The content of Igs in colostrum and milk is highly dependent on the animal species. The same holds for the relative proportion of the Ig classes (Figure 2). These species differences are adaptations to the reproductive strategies of the animals and the degree of offspring maturation at birth. These adaptations have several consequences both for the composition of Igs in colostrum and milk, and for the role of colostrum (Walter L. Hurley and Theil, 2011). The profile of total Igs in human colostrum is similar to that found in milk, where the IgA level is high in both colostrum and milk (88–90% of total Ig). This is contrast to the bovine mammary secretions where the high concentration of IgG in colostrum declines rapidly with successive milking's (Figure 2).

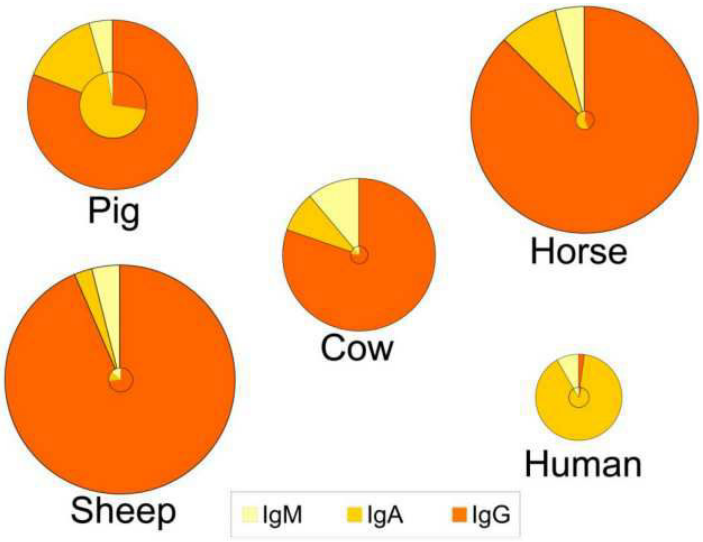

Figure 2. Relative distribution of IgA, IgM and IgG in colostrum (outer circle) and in milk (inner circle) of five species. The relative size of the circles represents the overall concentration of total Igs found among the species and the concentrations in colostrum *vs*. milk. Data compiled and calculated from: cow and sheep; human and pig; and mare (Walter L. Hurley and Theil, 2011).

Lysozyme (EC 3.2.1.17) exerts its antimicrobial activity by the hydrolysis of glycosidic bonds of mucopolysaccharides in bacterial cell walls (Clare *et al.*, 2003; Fox and Kelly, 2006), and it is one of the most extensively studied antibacterial milk proteins (López Expósito and Recio, 2006). The enzyme hydrolyses β-1.4-glycosidic linkages between N-acetylmuramic acid and N-acetylglucosamine, resulting in lysis of the bacterial cell wall. The amino acid content of the bovine protein is significantly different from that of both human and egg white lysozyme, and the levels of the enzyme vary considerably between the species (Clare *et al.*, 2003; López Expósito and Recio, 2006). The enzyme is active against a number of Gram-positive and Gram-negative bacteria; however, the severity of this reactivity does vary. Gram-negative microorganisms are generally inactivated by the action of this protein, whereas, Gram-positive microorganisms are typically inhibited only in their growth (Clare *et al.*, 2003). Lysozyme may work synergistically with lactoferrin and Igs in antimicrobial functions (Fox and Kelly, 2006; Zagorska and Ciprovica, 2012).

1.3. Human milk and its substitutes

Milk is a physiologically and nutritionally balanced secretion, suitable to every requirement of newborns. Hence, human milk is the best food for babies with healthy mothers who have appropriate nutritional stores (Criscione *et al.*, 2009; Shamsia, 2009). Breast milk of a healthy and well-nourished woman is the best reference for nutritional requirements during the early neonatal period. Commercial infant formulas have been developed as nutritional substitutes for breast milk and mimic, where possible, the levels and types of proteins, fatty acids, sugars, vitamins, minerals, and other nutrients present in human milk. Despite this, differences in response to infection, development of allergies and atopic diseases have been reported in formula-fed compared with breast-fed infants (Paolo Polidori *et al.*, 2009).

When breast-feeding is not possible or after weaning, finding an adequate alternative nourishment becomes mandatory (Barłowska *et al.*, 2011; Criscione *et al.*, 2009; El-Agamy, 2007; Paolo Polidori *et al.*, 2009). Cow's milk is widely used as a substitute for human milk but, in an increasing number of cases, it can lead to an abnormal immunological response, such as allergy to Cow's Milk Proteins (CMP) (Barłowska *et al.*, 2011; Bidasolo *et al.*, 2012; Criscione *et al.*, 2009; El-Agamy, 2007; Paolo Polidori *et al.*, 2009; Vincenzetti *et al.*, 2011). In this respect, Cow's Milk Protein Allergy (CMPA) is the most common food allergy, affecting about 3% of children in the first three years of life (Businco *et al.*, 2000; Criscione *et al.*, 2009; D'Auria *et al.*, 2005; Di Bella *et al.*, 2012; Monti *et al.*, 2007; Tesse *et al.*, 2009). The milk proteins mainly responsible for the allergy are α- and β-caseins, followed by β-lactoglobulin and α-lactalbumin to a lesser extent (El-Agamy, 2007; Vincenzetti *et al.*, 2012). Clinical manifestations of CMPA are atopic dermatitis, urticarial/angioedema, gastrointestinal symptoms, and less frequently, respiratory disorders, such as wheezing and asthma (Tesse *et al.*, 2009). Successful therapy depends on completely eliminating CMP from the child's diet (El-Agamy, 2007; Monti *et al.*, 2007; Tesse *et al.*, 2009).

For human beings cow's milk represents the most common feeding during the infant weaning, but also the first allergen in life (Bylund, 1995; Vincenzetti *et al.*, 2008). The European Academy of Allergy and Clinical Immunology distinguishes allergy from intolerance. Allergy is an adverse reaction to food with an involvement of the immune system, intolerance is an adverse reaction to food that does not involve the

immune system, does not reply to a precise and single fault and shows different symptoms (Monti *et al.*, 2007; Paolo Polidori *et al.*, 2009), such as abdominal symptoms and chronic diarrhea after ingestion of milk (Uniacke-Lowe *et al.*, 2010). Lactose intolerance is not a disease, 70% of the world population is lactose-intolerant, and adverse effects of lactose intolerance occur at higher levels of milk consumption than milk protein allergy (Uniacke-Lowe *et al.*, 2010).

Hypoallergenic alternatives to breast milk have been proposed through the years and include extensively hydrolyzed formulas (eHF), amino acid formulas (AAF) and soy formulas (SF) (Criscione *et al.*, 2009; Restani *et al.*, 1999).

Therapeutic formulas based on extensively hydrolyzed proteins should represent the preferred choice in the treatment of CMPA (D'Auria *et al.*, 2005). They have a good nutritional value, but are not tolerated by all patients with CMPA. Besides, they are quite expensive, present poor palatability, and may contain residual allergenic epitopes (Criscione *et al.*, 2009; Monti *et al.*, 2007; Tesse *et al.*, 2009). AAF are the only breast milk substitutes considered to be non-allergenic, but they have an unpleasant bitter taste, and should be considered in severe clinical manifestations of food allergy or when eHF are not effective (Criscione *et al.*, 2009; Tesse *et al.*, 2009). As an alternative to hydrolysed and hypoallergenic cow's milk formulas, soy bean-derivated formulas and milk from mammalian species other than cows have been used (D'Auria *et al.*, 2005). SF, although providing a moderate palatability and in many cases good nutritional benefits, are not generally recommended before the age of 6 months for infants with IgE-associated CMPA symptoms (Caffarelli *et al.*, 2010; Criscione *et al.*, 2009; Monti *et al.*, 2007). For these reasons, other mammalian milks have been proposed as alternatives to hydrolyzates of CMP and soy bean formulas (D'Auria *et al.*, 2005; Tesse *et al.*, 2009).

In the last few years, milk from non-bovine mammals (e.g.: goat, donkey, mare, and camel) has been studied to identify the best natural substitute for human milk (Criscione *et al.*, 2009). Goat and sheep milks are contraindicated as their proteins have shown extensive cross-reactivity with CMP both *in vitro* and *in vivo* (Monti *et al.*, 2007). Mare's milk appears to be more promising, as in composition it is much closer to human milk than to cow's milk and it has been found to be tolerated by some children with severe IgE-mediated CMPA. However, its availability is limited and collection is difficult (Monti *et al.*, 2007). Donkey milk was found to be a valid alternative to both

IgE-mediated and non-IgE-mediated CMPA, with favourable effects on palatability and weight-height gain in an increasing number of clinical trials (Criscione *et al.*, 2009; Vincenzetti *et al.*, 2011). Compared to ruminants' milk, donkey milk has been less studied in the past, but in the last years research interest and capital investment in donkey milk have increased. This is because it is similar to human milk in terms of general composition and particular protein fraction (Barłowska *et al.*, 2011; Di Bella *et al.*, 2012; Nazzaro *et al.*, 2010; Paolo Polidori *et al.*, 2009; Tafaro *et al.*, 2007; Vincenzetti *et al.*, 2012; Zhang *et al.*, 2008), as is shown in Tables 1 and 2. Donkey milk is the major substitute of human milk and its components are very powerful in the mitigation of allergy state and inflammation (Jirillo *et al.*, 2010). Additionally, donkey milk is a strong vasodilator, making it potentially useful in the prevention of atherosclerosis, and has been shown to exert an *in vitro* suppressing action against human lung tumours (Nazzaro *et al.*, 2010). Recent reports confer anti-tumour and anti-proliferative activity to donkey milk (Bidasolo *et al.*, 2012). This milk receives increasing research interest in Europe, mainly owing to its attractive nutrient and functional contents; as its chemical composition is similar to human milk, donkey milk can be used as an alternative for infants who show intolerance to bovine milk (Cunsolo, Costa*, et al.*, 2007; Zhang *et al.*, 2008). Vincenzetti *et al.* (2012) confirmed on their study that the nutritional characteristics of the protein fractions of donkey milk and the possibility of using this milk in feeding children with CMPA, including children with multiple food allergies due to low amount of caseins (Vincenzetti *et al.*, 2012).

In the last few decades, social, economic, safety, and nutritional factors have had their impact on food markets and the use of some new commercial food preparations which claim to possess beneficial dietetic and therapeutic properties is emerging more and more rapidly. Amongst the new foods, particular interest is addressed to donkey milk, already used as a replacement for human breast milk for infants affected by multiple food allergies and intolerances (Coppola *et al.*, 2002).

Table 1. Average milk composition (g/Kg) in different species. Adapted from Nikkhah (2012); P. Polidori *et al.* (2009).

Component	Cow		Donkey		Human	
	Nikkah (2012)	Polidori *et al*. (2009)	Nikkah (2012)	Polidori *et al*. (2009)	Nikkah (2012)	Polidori *et al*. (2009)
Fat	40	35-39	11	3-18	40	35-40
Protein	34	31-38	17	15-18	19	9-17
Lactose	48	48[a]	66	58-74	65	63-70
Minerals	7	-	4	-	2	-
Solids, non-fat	90	-	92	-	73	-
Total solids	133	125-130	102	88-117	121	117-129
Cholesterol	140	-	22	-	200	-
Calcium	1200	-	680	676.6[b]	320	34[c]
Phosphorus	930	-	500	487.0[b]	140	16[c]
Saturated Fatty Acids	24	-	4	-	18	-
Monounsaturated	11	-	2	-	16	-
Polyunsaturated	1	-	4	-	5	-
Ash	-	0.7-0.8	-	0.3-0.5	-	0.2-0.3
pH	-	6.6-6.8	-	7.0-7.2	-	7.0-7.5

[a] (Fox and McSweeney, 1998); [b] (mg/Kg) (Rathore *et al.*, 2011); [c] (mg/100 mL) (Rathore *et al.*, 2011); "-" means not reported data

Table 2. Comparison of protein content (g/L) of cow's, donkey and human milk. Adapted from Vincenzetti *et al.* (2012) and Salimei and Fantuz (2012).

	Cow		Donkey		Human	
	Vincenzetti *et al*. (2012)	Salimei *et al*. (2012)[a]	Vincenzetti et al. (2012)	Salimei *et al*. (2012)[a]	Vincenzetti *et al*. (2012)	Salimei *et al*. (2012)[a]
Lactoperoxidase (mg/L)	30-100	-	0.11	-	0.77	-
Lactoferrin	0.1	1.6	0.8	4.48	0.3-4.2	26.6
Lysozyme	trace	trace	1.0	21.0	0.12	5.5
β-lactoglobulin	3.3[b]	50.8	3.75[b]	29.8	Absent[b]	Absent
α-lactalbumin	1.6[b]	19.0	1.8[b]	22.6	2.2[b]	40.3
Caseins	27.2[b]	-	6.6[b]	0.87[c]	5.8[b]	0.63[d]
Whey protein	4.5[b]	-	7.5[b]	0.68[c]	8.0[b]	1.31[d]
Immunoglobulins	-	12.7	-	11.5	-	15.5

[a] (%); [b] (Vincenzetti *et al.*, 2008); [c] (g/100 g) (Rathore *et al.*, 2011); [d] (%) (Rathore *et al.*, 2011); "-" means not reported data

2. Donkey Milk

2.1. The donkey

The donkey (*Equus asinus*, order Perissodactyla, family Equidae and genus *Equus*) is a domestic animal belonging to the equine family, which includes horses, zebras and mules. Donkeys progenitor was the small gray donkey of northern Africa (*Equus africanus*) domesticated around 4,000 BC on the shores of the Mediterranean Sea (Herrouin *et al.*, 2000; P. Polidori *et al.*, 2009; Polidori *et al.*, 2008; Salimei and Fantuz, 2012). The donkey worked together with humans for centuries and contributed to the development of various civilizations; the most common role was for transport, and still remains an important work animal in the poorer regions (Polidori *et al.*, 2008). Donkey is a complex creature, capable of many moods. It can be friendly, affectionate, patient, independent, and especially intelligent. This animal has a keen sense of curiosity, and an incredible memory, but it is also stubborn in an original way. Donkey has a slow gait, and is most active in the evening. The size varies considerably from 0.9 to 1.6 m. The gestation is approximately of 12 months, and life is of 25-30 years (Paolo Polidori *et al.*, 2009).

Even today, in some countries such as Egypt, Nigeria, Mali, Niger, and Sudan donkeys are more common than horses. In these countries, and in other parts of the world, like the Middle East and/or Asia, donkeys are still an important means of transport and can survive, reproduce and, produce meat and milk in difficult environmental conditions (Polidori *et al.*, 2008). Donkeys remain essential for rural economies in semiarid and mountainous areas of the world.

The donkeys can be used in different ways, many of which are similar to those of the horse, like trekking, meat production, pet therapy and brain gym, which is a program of physical movements that enhance learning and performance in all areas. The European Union protects this species through different policies, thus providing financial incentives to raise donkeys. The Food and Agriculture Organization (FAO) considers the donkey species in danger of extinction (Giosue *et al.*, 2008), and in recent years the necessity to protect a species in danger of extinction and to reassess the function of donkey milk has led to increased interest in this species (Failla, 2008). Although, donkey milk production has recently become the main interest. Donkeys are rustic

animals with few requirements. They adapt easily, which means that in most cases they can be reared in a semi-wild state, thus reducing initial investment costs and expenditure (Failla, 2008).

However, because of limited donkey milk production, the quantities available for human consumption are, in realistic scales, still inadequate (Nikkhah, 2012). Although Equidae dairy production and research data are limited, the emerging knowledge and perspectives should fuel more research that will base commercial investments in breeding dairy Equidae and manufacturing value-added dairy products (Nikkhah, 2012).

2.2. Donkey milk

The use of dairy products from donkeys was known in the Roman era, and for a long time donkey milk was recognized as a common remedy. In the late nineteenth century, donkey milk was successfully used for feeding orphaned infants in France, as reported by D'Arval (1912) (Salimei and Fantuz, 2012).

In the second half of the last century, donkey population dramatically decreased in Europe, which can mainly be attributed to the substitution of work animals by machinery. However at the beginning of the 21st century, the number of donkeys in the south of Europe is increasing, because of the great interest in donkey milk, whose composition resembles that of human milk more than bovine and/or goats milk (P. Polidori *et al.*, 2009).

Traditionally, donkey milk has been recognized by its legendary cosmetic and therapeutic properties (Bidasolo *et al.*, 2012; Cunsolo, Costa*, et al.*, 2007; Cunsolo *et al.*, 2011; Herrouin *et al.*, 2000). These properties were well known not only by the Queen of Ancient Egypt Cleopatra but also by Roman Emperor Neron's wife Poppea Sabina, who used to take donkey milk baths to keep a smooth and young skin. Hippocrates (460–370 BC), the father of medicine, prescribed donkey milk for numerous purposes, such as liver troubles, infectious diseases, fever, poisonings, edemas, asthma, etc. (Bidasolo *et al.*, 2012; Cunsolo *et al.*, 2011). Nowadays, donkey milk is widely produced and consumed in Asia, Africa, and Eastern Europe, with China having the largest donkey stock worldwide, followed by Pakistan and Ethiopia (Bidasolo *et al.*, 2012; Nazzaro *et al.*, 2010; Šarić *et al.*, 2012). Likewise, in some

Western Europe countries, especially in Italy, the number of donkey breeding farms has notably increased in recent years (Bidasolo *et al.*, 2012). In fact, in the Italian popular tradition of the last centuries, donkey milk was commonly used to feed infants when the maternal one was not sufficient and actually it seems to attract the consumers interest (Uniacke-Lowe *et al.*, 2010; Vincenzetti *et al.*, 2012).

Donkey milk is very different from milk of other species traditionally used for human feeding such as cow's, goat, and sheep milks, showing a closer similarity to human milk (Brumini *et al.*, 2013; Simos *et al.*, 2011), as shown in Table 1 and 2. Recent studies have indicated that donkey milk may be a promising food for children affected by CMPA or multiple food intolerance (Carroccio *et al.*, 2000; Iacono *et al.*, 1992; Lionetti *et al.*, 2012; Tesse *et al.*, 2009) and for the elderly, because of its ability to up-regulate the immune response (Amati *et al.*, 2010; Simos *et al.*, 2011; Tafaro *et al.*, 2007). Today, it has been rediscovered for its characteristics such as high digestibility, elevated nutrient value, and physiological properties (Di Bella *et al.*, 2012). Donkey milk is a nutraceutical food, which responds to special needs of children, adults and elderly. It is a unique food with high commercial value (Di Bella *et al.*, 2012).

The properties of equine milk differ from those of other mammals in many ways that include important differences in nutritional value; moreover, its composition does not permit the production of cheese due to the high content of whey proteins. These proteins represent 35-50% of the nitrogen fractions, whereas in cow's milk they account for only 20% (Table 1) (Chianese *et al.*, 2012; Cunsolo, Saletti*, et al.*, 2007; Paolo Polidori *et al.*, 2009). Generally and as shown in Table 1, in comparison with cow´s milk, donkey milk contains less fat, protein, inorganic salts, but more lactose; its content is not only closer to human milk, but it also considered to be highly digestible, palatable, and rich in essential nutrients (Tidona *et al.*, 2011).

Lactose is the most representative element of donkey milk, being its value 66 g/Kg of milk (Table 1), the same as in the human milk, being higher if compared to cow's milk. In particular, lactose represents an ideal substrate for the intestinal flora development in humans and, technologically, can be utilized in the preparation of probiotic solution for human use (e.g. inflammatory bowel disease) (Tafaro *et al.*, 2007). Some authors have also suggested using donkey's milk for probiotic purposes, since it has several beneficial qualities, such as low microbial activity (about 4×10^4

CFU/mL) and a high amount of lysozyme (Di Bella *et al.*, 2012; Nikkhah, 2012) which stabilizes pH (Nikkhah, 2012), and it proved to be a good growth medium for probiotic *Lactobacilli* strains, because of the lysozyme and lactose high content (Nikkhah, 2012; Vincenzetti *et al.*, 2008).

The high content of lactose is also responsible for the good palatability and facilitates the intestinal absorption of calcium, that is essential for infant bone mineralization (Vincenzetti *et al.*, 2008). Besides, the high lactose value facilitates the manufacture of fermented products and drinks (Nikkhah, 2011). Lactose is thought to have a major effect on bone mineralization during the first few months after birth as it stimulates the intestinal absorption of calcium. With high lactose content similar to human milk, donkey milk would seem to be suitable for infant nutrition, especially as lactose intolerance is uncommon in infants and children under two years of life (Uniacke-Lowe *et al.*, 2010). In addition, because of its high lactose content, donkey milk could be placed amongst the new generation of fermented milk drinks, as is koumiss, deriving from mare's milk, and would allow for an effective combination of the advantageous properties of the raw ingredient with lactic acid bacteria. These observations suggest that the use of donkey milk in human diet deserves reconsideration by food science experts. The elaboration of such efforts would also contribute to the preservation of animal biodiversity, balancing out the decreasing trend widely present in donkey population and particularly in those breeds which are threatened by extinction (Coppola *et al.*, 2002).

The low allergenicity of donkey milk is mainly due to the low casein content (Table 2) that is characteristic of this milk and very close to the casein content of human milk (Nikkhah, 2011; Vincenzetti *et al.*, 2011). About casein composition of donkey milk, recent works (Bertino *et al.*, 2010; Criscione *et al.*, 2009; Vincenzetti *et al.*, 2008) showed the presence of α_{s1}- and β-caseins. Caseins are present in different phosphorylated and glycosylated forms, while κ-casein and α_{s2}-casein are present in this milk but in very small amounts, differently from dairy cow's milk (Vincenzetti *et al.*, 2011).

Donkey milk shows a lower fat content compared to human milk (Table 3), consequently presenting a reduced energetic value, which may contribute to a better cardiovascular health (Piccione *et al.*, 2008). The large number of fatty acids present in the lipid fraction of milk makes it one of the most complex naturally occurring fats

(Figure 3). In donkey milk, the lipid fraction is the most important in terms of dietary application (Chiofalo *et al.*, 2003; Tafaro *et al.*, 2007). Donkey milk significantly differs from human and cow's milk for its lower total lipid content (0.94%, *vs.* 3.6 and 3.8%, respectively) which results in its lower caloric content (408 Kcal/L, *vs.* 690 and 660 Kcal/L, respectively) (Gastaldi *et al.*, 2010). Also, the lipid fraction is similar to that of human milk and is characterized by high levels of linolenic and linoleic acids (Salimei *et al.*, 2004). Its addition in diet may be useful for the treatment of some atopic dermatitis (Vincenzetti *et al.*, 2008). Therefore, donkey's milk could be used as a nutritional medicine for children suffering from this pathology (Horrobin, 2000). The elevated content of the ω-3-fatty acids (typical fish oil constituents) supports the use of donkey milk as an effective functional food in the prevention of cardiovascular disease, autoimmunity, and chronic inflammatory processes (Tafaro *et al.*, 2007).

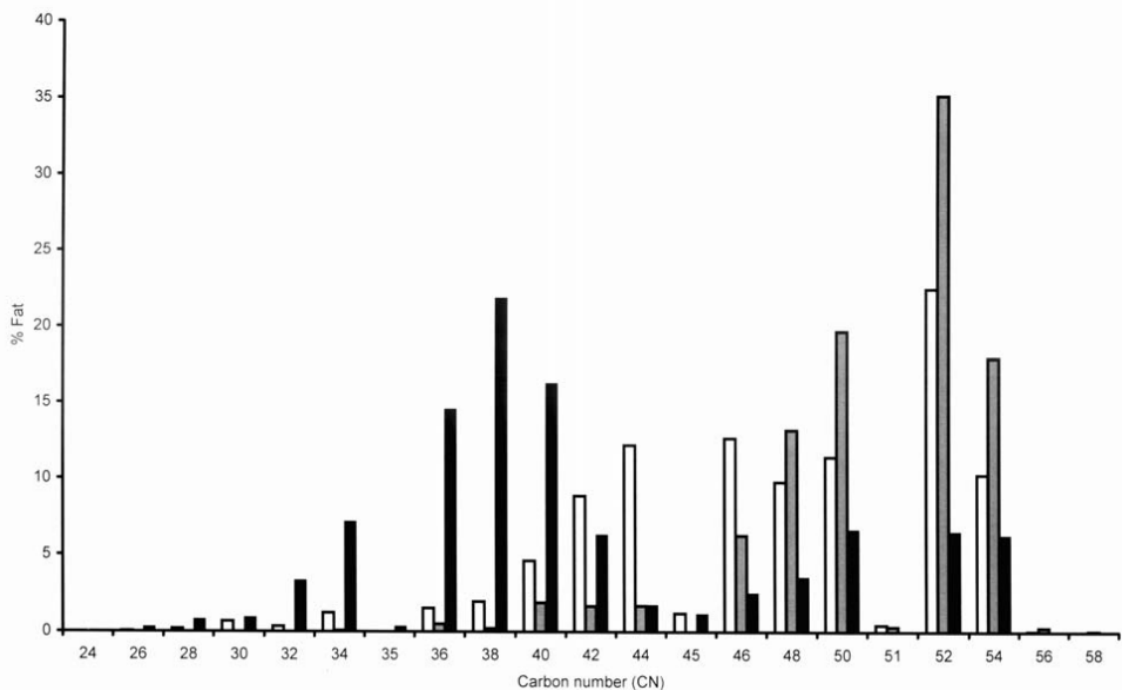

Figure 3. Distribution of triacylglycerols (TAGs) within different carbon number (CN) groups in donkey, human and cow's milk. DM: white, HM: grey, CM: black. Adapted from Gastaldi *et al.* (2010).

Table 3. Fatty acid composition of milk samples. Adapted from Gastaldi *et al.* (2010).

Name		**D. M.**	**H. M.**	**C. M.**	**D. M.**	**H. M.**	**C. M.**
		g/100 g of fat			mg/100 mL of milk		
Butyrric acid	C4:0	0.57	0.01	3.77	5.34	0.38	1.37
Caproic acid	C6:0	1.16	0.02	2.32	10.9	0.77	84.4
Caprylic acid	C8:0	2.33	0.10	1.39	21.8	3.85	50.6
Capric acid	C10:0	6.58	0.15	3.34	61.6	5.77	122
Undecanoic acid	C11:0	0.67	0.01	0.13	6.27	0.38	4.73
Lauric acid	C12:0	6.99	6.54	4.15	65.4	252	151
Tridecanoic acid	C13:0	3.72	0.02	0.19	34.8	0.77	6.92
Myristic Acid	C14:0	6.67	5.38	11.3	62.4	207	411
Myristoleic Acid	C14:1	0.21	0.34	0.78	1.97	13.1	28.0
Pentadecanoic Acid	C15:0	0.3	0.23	0.36	2.81	8.85	13.1
Palmitic Acid	C16:0	26.3	20.0	28.8	246	770	1050
Palmitoleic Acid	C16:1	2.25	3.10	1.55	21.1	119	56.4
Margaric Acid	C17:0	0.21	0.28	0.53	1.95	10.8	19.3
Stearic Acid	C18:0	2.68	6.15	14.2	25.1	237	517
Oleic Acid	C18:1 n-9	17.0	32.6	20.7	159	1250	753
Octadecenoic Acid	C18:li	1.14	7.99	2.10	10.7	307	76.4
Linoleic Acid	C18:2 n-6	9.50	12.2	2.44	88.9	469	88.8
a-Linolenic Acid	C18:3 n-3	7.25	1.14	0.48	67.9	43.9	17.5
c-Linolenic Acid	C18:3 n-6	0.14	0.05	0.18	1.31	1.92	6.35
Arachidic Acid	C20:0	0.11	0.23	0.18	1.03	8.85	6.55
Eicosaenoic Acid	C20:1 n-11	0.33	0.05	0.14	3.09	1.91	5.10
Eicosadienoic Acid	C20:2 n-6	0.33	0.39	0.0	3.10	15.0	0.0
Eicosatrienoic Acid	C20:3 n-3	0.11	0.05	0.0	1.03	1.93	0.0
Arachidonic Acid	C20:4 n-6	0.07	0.59	0.22	0.66	22.7	8.01
Eicosapentaenoic Acid	C20:5 n-3	0.26	0.02	0.06	2.43	0.76	2.18
Docosanoic Acid	C22:0	0.05	0.38	0.05	0.47	14.6	1.82
Docosapentaenoic Acid	C22:5 n-3	0.07	0.18	0.18	0.66	6.93	6.42
Docosahesaenoic Acid	C22:6 n-3	0.28	0.40	0.05	2.62	15.4	1.82

(D.M. – Donkey milk; H.M. – Human milk; C.M. – Cow's milk)

The values of mineral composition in donkey milk are very close to those of human milk (Table 1). In particular, calcium and phosphorus levels are higher than in human milk, thus contributing to a better activation of immune cells, mostly in patients with immunodeficits, as previously indicated by Jirillo *et al*. (2003) (Tafaro *et al.*, 2007). Donkey milk total protein is low, very close to the values for human milk and thus, it does not produce an excessive renal load of solute (Vincenzetti *et al.*, 2008). The good intestinal absorption of calcium makes it suitable for bone mineralization in children and to prevent osteoporosis in the elderly (Di Bella *et al.*, 2012).

The major whey proteins in equine milk are β-lactoglobulin (β-Lg), α-lactalbumin (α-La), Igs, bovine serum albumin (BSA), lactoferrin (LF) and lysozyme (Barłowska *et al.*, 2011; Cunsolo, Saletti*, et al.*, 2007; Herrouin *et al.*, 2000; Uniacke-Lowe *et al.*, 2010), which is similar to cow's milk. Except for β-Lg, all of these proteins are also present in human milk (Fantuz *et al.*, 2001; Nikkhah, 2011). However, the relative amounts of the whey proteins differ considerably between these species (Table 2). Comparatively to cow's milk, donkey milk contains less β-Lg and more α-La and Igs (Nikkhah, 2012; Tidona *et al.*, 2011), and the low allergenic protein content makes donkey milk a favourable candidate for the replacement of bovine milk in the diet of milk-allergic human infants (Piccione *et al.*, 2008). The main antimicrobial agent in donkey milk is lysozyme and to a lesser extent LF, which predominates in human milk (Barłowska *et al.*, 2011; Uniacke-Lowe *et al.*, 2010). Together, IgA, IgM, IgG, LF and lysozyme provide the neonate with immune and non-immune protection against infection, for example, prevent intestine infections in infants (Uniacke-Lowe *et al.*, 2010). The high lysozyme content found in donkey milk (Table 2) may be responsible for its low microbial load (Nazzaro *et al.*, 2010; Salimei *et al.*, 2004; Vincenzetti *et al.*, 2008) and could be useful to prevent intestine infections in infants (Vincenzetti *et al.*, 2011). Donkey milk is reported to have stronger inhibitory microbial activity than that of any other species (Malacarne *et al.*, 2002; Zhang *et al.*, 2008).

Protein characterization of the tested donkey milk by electrophoresis indicates lysozyme as the main antimicrobial agent but is also important in the cellular immunity (Qureshi and Enbergs, 2012). The reported concentration of 1.31 g/L by Šarić *et al.* (2012) is in accordance with results obtained by Vincenzetti *et al.* (2008). Recent studies also marked lysozyme as the most abundant antimicrobial agent in donkey milk (Coppola *et al.*, 2002; Vincenzetti *et al.*, 2008; Zhang *et al.*, 2008). A synergistic

activity of lysozyme and LF could also contribute to antibacterial activity of donkey milk (Šarić *et al.*, 2012). The raw donkey milk antimicrobial potential and long shelf-life could possibly be attributed to the presence of milk proteins and other antimicrobial agents that probably act in a synergistic manner (Brumini *et al.*, 2013; Šarić *et al.*, 2012).

2.3. Bioactive proteins in donkey milk

Donkey milk has been used successfully as an alternative food for infants with food allergies, e.g. CMPA, a common food allergy in childhood (Salimei and Fantuz, 2012), ranging between 2 and 7.5% of the infant population. Food allergies are one of the most frequent causes of malabsorption and growth deficiency in unweaned infants in the first few months after birth (Iacono *et al.*, 1992). Patients with CMPA often show allergy to other foods including powdered milk containing soy or hydrolyzed proteins. In children with CMPA, when it is not possible to breast-feed, the clinical use of donkey milk is considered, since several studies have demonstrated the high similarity of donkey milk to human milk. Furthermore, studies performed using the serum of children with CMPA and milk proteins from other mammalian species evidenced a weak cross-reactivity with milk from donkey (Vincenzetti *et al.*, 2012). In particular, the first clinical evidence by Iacono *et al.* (1992) suggests that infants with food allergy could tolerate donkey milk (210 – 250 mL milk/Kg body weight/day) (Salimei and Fantuz, 2012).

➔ Case reports, adapted from Iacono *et al.* (1992):

It is well known that CMP represents only some of the many possible allergens that can trigger food hypersensitivity reactions. In fact, there are well-documented reports in the literature of cases of allergy to soya, hydrolyzed casein, rice, and other foods. Furthermore, these rarer food hypersensitivities are known to be more frequent in patients initially allergic to cow's milk proteins. Therefore, in these cases, there is a multiple food allergy, which is a difficult condition to treat.

Iacono *et al*. (1992) reports the clinical data of 9 patients with multiple food allergies, and the onset of symptoms in all patients occurred during the first month after

birth and in four of them immediately after birth. In these latter cases the first sign of food intolerance was that there was no, or very little, weight increase. The results obtained by these authors are shown in Table 4.

The introduction of donkey milk into the diet caused no allergic reactions in the infants, and this milk was administered at a dose of 250 mL/Kg/day. Therefore, it is not necessary to dilute donkey milk before feeding it to infants; this represents a considerable advantage considering the lower fat, and obviously caloric, content of donkey milk compared to cow's milk (Table 1).

After the introduction into the diet, no fecal mucus was observed, plus occult fecal blood tests were negative. No patient suffered from vomiting and there were no respiratory or cutaneous allergic reactions. Laboratory tests showed the absence of eosinophilia and fecal eosinophils in all patients. After 24 h of donkey milk introduction into the human diet, the infants were visibly more vivacious and successively a considerable weight increase was observed. The donkey milk was the only food given to these babies for an average period of 25 days (range of 15-35 days); at a later stage, cereal flours, olive oil, and meat were carefully reintroduced. The clinical follow-up confirmed the absence of symptoms in the patients and the negative objective examinations. Subsequent clinical studies showed interesting results of donkey milk tolerability (Table 5), when breastfeeding is not possible or not advisable and hypoallergenic milk formulae are not tolerated (low palatability, cross-reactions, etc.) (Salimei and Fantuz, 2012).

Table 4. Relationship between symptoms and foods in nine nurslings affected with multiple food intolerance. Adapted from Iacono *et al.* (1992).

Case	Age at onset	Age at hospitalization (days)	Cow's milk	Soy milk	Hydrolyzed milk	Hypoallergenic diet
1, m	Since birth	30	Failure to thrive, vomiting (1-15)	Diarrhea, vomiting (16-30)	Vomiting, regurgitation (31-37)	Diarrhea (38-48)
2, m	12 days	49	Diarrhea, vomiting (1-15)	Diarrhea (16-48)	Shock (49)	Bloody diarrhea (50-55)
3, m	20 days	60	Diarrhea, vomiting (1-15)	Bloody diarrhea (31-42)	Urticate (43-60)	Diarrhea (61-70)
4, f	Since birth	26	Failure to thrive, vomiting (1-19)	Diarrhea, vomiting (20-26)	Diarrhea, vomiting (27-31)	Diarrhea (32-36)
5, m	11 days	47	Failure to thrive (1-25)	Diarrhea (26-34)	Diarrhea, vomiting (35-47)	Bloody diarrhea (48-51)
6, f	7 days	53	Diarrhea, vomiting (1-13)	Bloody diarrhea (14-49)	Bloody diarrhea (50-53)	Diarrhea (54-59)
7, m	Since birth	79	Failure to thrive (1-56)	Diarrhea, vomiting (57-64)	Failure to thrive (65-79)	Diarrhea, vomiting (80-86)
8, f	Since birth	74	Failure to thrive, vomiting (1-60)	Diarrhea (61-74)	Failure to thrive, diarrhea (75-85)	Diarrhea, vomiting (86-88)
9, m	20 days	52	Diarrhea, vomiting (1-48)	Bloody diarrhea (49-50)	Bloody diarrhea (51-52)	Diarrhea (53-59)

Numbers in parenthesis indicate the days of administration of different foods since birth. m, Male; f, female. Hypoallergenic diet composed of ground rice cream, homogenized chicken, and olive oil.

Table 5. Clinical studies in tolerability of donkey milk on children with food allergies. Adapted from Salimei *et al*. (2012).

Author	**Children's age**	**Experimental conditions**	**Tolerability (%)**
Iacono *et al*. (1992)	26 – 79 days	9 Unweaned children with multiple food allergies	100
Carrocio *et al*. (2000)	10 days – 9 months	21 children with food allergies and hydrolyzed proteins intolerant	86
Vita *et al*. (2007)	6 months – 3 years	26 CMPA and atopic dermatitis children	88
Monti *et al.* (2007)	12 – 149 months	46 CMPA children intolerant to common CMP substitute	82.6
Tesse *et al*. (2009)	6 months – 11 years	30 children with mild to moderate CMA	96

Vita *et al.* (2007) showed that the rate of tolerability of donkey milk in patients with atopic dermatitis and CMPA was 88%, and this milk improved child's eczema (Vita *et al.*, 2007). Similarly, Monti *et al.* (2007) have documented that the tolerability of donkey milk was 82.6% in their selected cohort of children with CMPA (46 children), without other alternative to the use of common CMP substitutes (Tesse *et al.*, 2009), and 38 children (82.6%) liked and tolerated donkey milk at the challenge and for the entire duration of follow-up (Monti *et al.*, 2007). Tesse *et al.* (2009) investigated tolerance and nutritional adequacy of donkey milk in 30 children with CMPA from Southern Italy (Tesse *et al.*, 2009), and reported that donkey milk was tolerated by 25 of the children (96%) either with the IgE- and the non-IgE-mediated CMPA. All enrolled subjects found it acceptable due to its palatability, and did not interrupt the study. Donkey milk has been considered an alternative to cow milk considering that its protein composition is similar to human milk (Tesse *et al.*, 2009).

The flavour and appearance of equid milk have been found to be attractive to children, which is of particular importance to young consumers (Salimei and Fantuz, 2012). Children with CMPA fed supplemented donkey milk showed significant increases in weight and other growth related parameters (Monti *et al.*, 2007; Tesse *et al.*, 2009).

Nutrition is also considered crucial in the immune recovery of elderly consumers and equid milk may be an interesting food also for this consumer group. Due to its immunological activities, raw donkey milk, and fermented derivatives are, in fact, considered useful in the prevention of atherosclerosis (Chiofalo *et al.*, 2006) and have the ability to up-regulate the immune response of healthy elderly consumers (Jirillo *et al.*, 2010).

2.4. Microbial profile of donkey milk

Milk is a good medium for the growth of many microorganisms, since it contains the necessary nutrients and provides a suitable physical environment (Zhang *et al.*, 2008). Donkey milk contains a low microbial load, being only 4 log CFU/mL (Chiavari *et al.*, 2005; Coppola *et al.*, 2002; Zhang *et al.*, 2008). This can be attributed to the antimicrobial substances present in donkey milk.

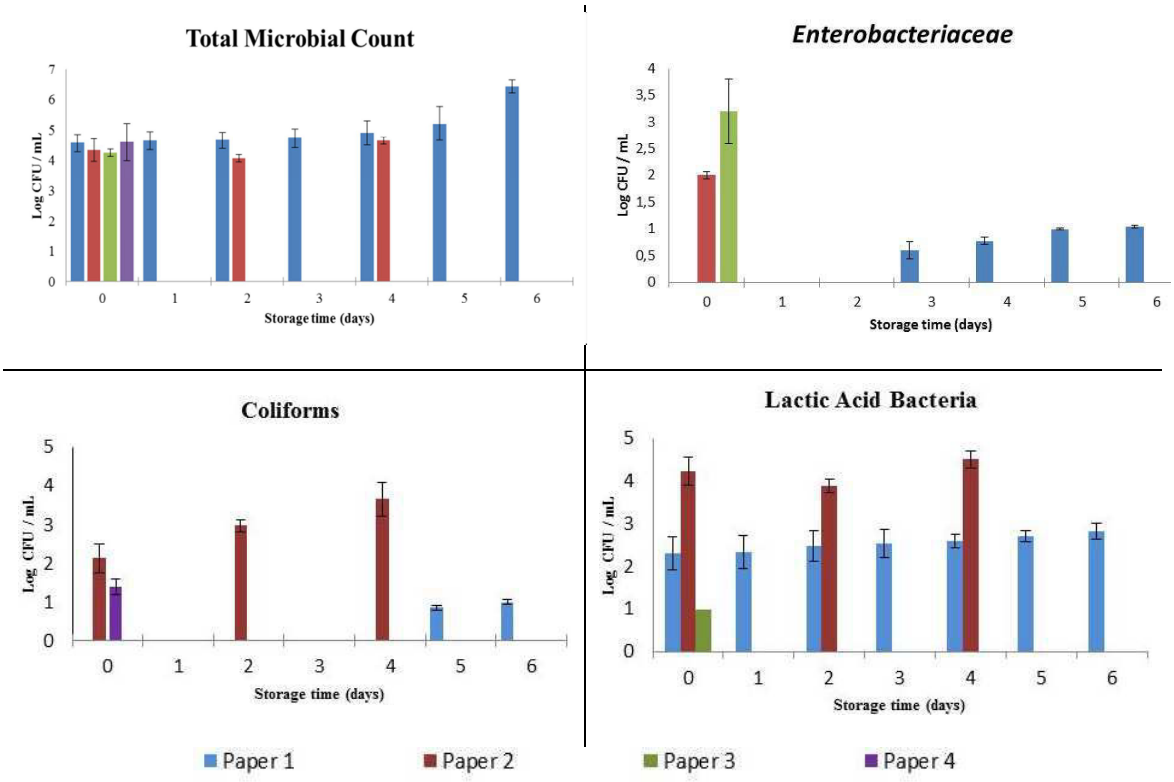

Figure 4. Compilation of donkey milk microbiological quality data of analyses of Total Microbial Count, *Enterobacteriaceae*, Total Coliforms and Lactic Acid Bacteria during storage at 4 °C by several authors. Paper 1 (Šarić *et al.*, 2012), Paper 2 (Zhang *et al.*, 2008), Paper 3 (Chiavari *et al.*, 2005) and Paper 4 (Coppola *et al.*, 2002).

According to Figure 4, Zhang *et al.* (2008) showed that the initial microbial count, lactic acid bacteria and coliforms on donkey milk were 4, 4 and 2 log CFU/mL, respectively. These authors did not observe significant changes of total microbial count after 96 h of storage. Sanjuan *et al.* (2003) obtained an increase of 1 log CFU/mL in total microbial count after 96 h in ovine milk, which differs from the obtained results by Zhang *et al*. (2008). Lactic acid bacteria were the predominant microorganisms during the storage, representing 79.4 and 74.1% at 0 h and after 96 h of storage, respectively. However, coliforms developed at 4 °C, increasing 1 log CFU/mL after 96 h of storage (Zhang *et al.*, 2008).

Usually, raw donkey milk contains a low microbial count (approx. 4.34 log CFU/mL) than the microbial load observed in bovine and ovine milk (7 and 5-7 log CFU/mL, respectively) (Chye *et al.*, 2004; Morgan *et al.*, 2003). The reason for the low microbial counts in donkey milk is due to the natural antimicrobial substances present in

it, such as the high lysozyme content (Guo *et al.*, 2007; Salimei *et al.*, 2004). The results obtained by Zhang *et al*. (2008) explained that refrigeration temperature might be an effective control of microorganism growth. But the rapid growth of coliforms at 4 °C should not be overlooked. Thus, it is important to improve the hygienic practices and establish the standards in donkey milk. On the other hand, the growth of lactic acid bacteria in donkey milk was not affected by these antimicrobial compounds (lysozyme), which demonstrates that donkey milk might be used as an interesting ingredient for many healthy fermented milk beverages.

Considering the numerous benefits of donkey milk, including its health-promoting characteristics and probiotic effects, Coppola *et al.* (2002) suggested the possibility of using donkey milk for probiotic purposes. The data obtained by these authors confirm that donkey milk is a possible basis for a fermented beverage as it contains several advantageous qualities, such as low microbial activity and high amounts of lysozyme, as well as being a vehicle for the consumption of probiotic bacteria (Coppola *et al.*, 2002; Pilla *et al.*, 2010).

Moreover, Chiavari *et al.* (2005) demonstrated that, in a fermented beverage with *Lactobacilli*, the lysozyme activity was found virtually unchanged in comparison with the initial values, even after a 30 days of shelf-life (Chiavari *et al.*, 2005). Pasteurized donkey milk has been inoculated with some strains of *Lactobacillus rhamnosus* (a microorganism with probiotic properties). *Lactobacillus rhamnosus* strains remained highly viable after 15 days of storage at 4 °C and at low pH (3.7-3.8). The high lysozyme content only partially influenced the growth of the strains tested without any significant effect on their acidifying activity (Coppola *et al.*, 2002; Salimei and Fantuz, 2012).

By analyzing Figure 3, Šarić *et al.* (2012) concluded that the total microbial count they obtained in donkey milk were similar to those obtained by other authors (4.2-4.6 log CFU/mL) (Chiavari *et al.*, 2005; Coppola *et al.*, 2002; Zhang *et al.*, 2008). Generally, raw donkey milk has lower total microbial counts comparatively to milk obtained from other species (Chye *et al.*, 2004; Morgan *et al.*, 2003). Šarić *et al*. (2012) obtained a lower count of lactic acid bacteria than that reported by Zhang *et al*. (2008). Šarić *et al*. (2012) concluded that the antimicrobial potential and long shelf-life of the raw donkey milk could possibly be attributed to the presence of milk proteins and other antimicrobial agents that probably act in synergistic manner. In this study, present in

high concentration, lysozyme could be marked as the main antimicrobial agent in donkey milk (AOAC, 2002; Šarić *et al.*, 2012).

3. Preservation methods

Heat treatment of milk may destroy heat-labile proteins, especially BSA and Igs, and change the antigenic properties of other whey proteins, such as β-Lg and α-La. On the other hand, caseins need severe heat treatment (121 °C for 20 min) to reduce their sensitizing capacity (Hill, 1994). Enzymatic treatment of milk proteins has resulted in products with unacceptable taste due to bitterness arising from the production of peptides and amino acids and such peptides may, in fact, be allergenic (Uniacke-Lowe *et al.*, 2010).

As sales of equine milk have increased considerably during recent years, research is now focused on the development of new products or new methods for extending its shelf-life, while maintaining some of the unique components of equine milk. The ability of milk to withstand relatively high processing temperatures is very important from a technological point of view. Whey proteins in equine milk are more thermal-stable than those of bovine milk, making equine milk less sensitive to thermal processing. However, heat treatment at 80 °C for 80 s still causes a 10-15% decrease in non-casein nitrogen, with a marked decrease evident when the temperature is increased above 100 °C (Uniacke-Lowe *et al.*, 2010).

LF and equine BSA appear to be the most sensitive but are not completely denatured until the temperature reaches 130 °C. β-Lg and α-La are almost completely denatured at temperatures over 130 °C and lysozyme at temperatures greater than 110 °C (Uniacke-Lowe *et al.*, 2010). The latter is in agreement with a study realized by Jauregui-Adell (1975), who found 68% residual lysozyme activity after heating at 82 °C for 15 min (Uniacke-Lowe *et al.*, 2010).

Lyophilization of donkey milk demonstrated that the nutritional characteristics of this product remained basically unchanged when compared to fresh milk; as a consequence, lyophilized donkey milk could be evaluated in further studies as a new dietetic food for infant nutrition to replace of breast milk. The high lysozyme content in donkey milk does not create a negative interaction with the possible supplementation with probiotic strains, giving therefore the opportunity of using donkey milk for the

production of probiotic beverages. Due to the recent interest in use donkey milk for the treatment of CMPA, the knowledge of the lyophilization effects of this product and the selection of other bacterial strains with probiotic properties should be deepened in order to investigate on other bionutritional parameters after a treatment than can help in supplying donkey milk on the market all over the year (Vincenzetti *et al.*, 2011).

In health food shops and some pharmacies in Western Europe, equine milk is sold frozen at -20 °C or as lyophilized milk capsules. It is claimed that many of these products relieve metabolic and intestinal problems while having a gut-cleansing effect coupled with "repair" of intestinal flora. Relief from stomach ulcers, high blood pressure, high cholesterol and liver problems are also reported and equine milk is recommended as an aid in the treatment of cancer patients. The recommended amount of equine milk is 250 mL per day. The use of equine milk in the production of cosmetics is relatively new and includes soaps, creams and moisturizers (Uniacke-Lowe *et al.*, 2010).

It is also recommended that hygienic practices and regulations, such as on-site pasteurization and implementation of HACCP, should also be introduced to facilitate the donkey milk production with high quality and safety (Zhang *et al.*, 2008). When donkey milk is produced hygienically, it is considered a valid substitute of hypoallergenic formulae but the low fat content must be appropriately balanced in the infant's diet. However, due their low fat content and unique fatty acid composition, donkey milk and their derivatives could become valuable foods for elderly consumers (Salimei and Fantuz, 2012).

Furthermore, the presence of endogenous bioactive compounds may help to explain the health-promoting properties of raw donkey milk. If confirmed by more in depth studies, the dairy equid species products could be explored in an agro-medical industry, where animal nutrition, management and milk should be carefully evaluated and regulated for safety reasons. In this regard, specific milk processing technologies are needed to improve the shelf-life and preserving natural attributes of equid milk (Failla, 2008; Salimei and Fantuz, 2012).

3.1. High pressure processing

Consumer trends and therefore food markets are changing and will change more in the future (Palou *et al.*, 2007). The food products quality and safety are two factors that influence the choices made by today´s consumers that are increasingly demanding (Fonberg-Broczek *et al.*, 2005; Hogan *et al.*, 2005). The modern consumer requires foods that are safe and nutritious, free from additives, taste good, and have a longer shelf-life (Evert-Arriagada *et al.*, 2012; McInerney *et al.*, 2007; Murchie *et al.*, 2005; Wright *et al.*, 2007). For these, the most important attributes of a food product are its sensory characteristics (e. g. texture, flavour, aroma, shape, and colour). These determine an individual preference for specific products and minor differences between brands of similar products can have a substantial influence on acceptability. A goal of food manufacturers is to develop and employ processing technologies that retain or create desirable sensory qualities or reduce undesirable changes in food due to processing (Hogan *et al.*, 2005). This trend has resulted, for many food products, in formulation and processing changes, which may previously have limited microbial growth (Murchie *et al.*, 2005).

For decades, various technologies have been used to preserve the quality and microbial safety of foods (Rodríguez *et al.*, 2003). Traditional preservation methods involve the use of heat (commercial sterilization, pasteurization, and blanching), preservatives (antimicrobials), and changes in the microorganisms environment, such as pH (fermentation), water availability (dehydration and concentration), or temperature (cooling and freezing) (Hogan *et al.*, 2005; Rodríguez *et al.*, 2003). The basis of these traditional methods involves reducing microbial growth and metabolism to prevent undesirable chemical changes in food, but probably the most common method of food preservation used today is thermal treatment (e.g. pasteurization and sterilization). Although heating food effectively reduces microorganisms levels such as bacteria, this process can alter the natural taste and flavour of food, and destroy vitamins (Hogan *et al.*, 2005).

Thermal pasteurization and sterilization are predominantly used in the food industry for their efficacy and product safety record (Lado and Yousef, 2002). However, excessive heat treatment may cause undesirable protein denaturation, non-enzymatic browning loss of vitamins, and volatile flavour compounds (Lado and Yousef, 2002).

Many methods of food preservation are used for ensuring microbiological safety (Fonberg-Broczek *et al.*, 2005), and non-thermal alternative technologies have been investigated intensively in the past 30 years (Lado and Yousef, 2002), among which high pressure processing (HPP) seems a very promising technique for food industry, as it offers numerous opportunities for developing new shelf-life, high nutritional value, and excellent organoleptic characteristics – minimally processed but safe for consumers (Fonberg-Broczek *et al.*, 2005; McInerney *et al.*, 2007; Mújica-Paz *et al.*, 2011).

Therefore, HPP is a promising non-thermal technique for food preservation that efficiently inactivates harmful pathogens and vegetative spoilage microorganisms, most commonly related to foodborne diseases (Garriga *et al.*, 2004; Heinz and Buckow, 2010; Mújica-Paz *et al.*, 2011; Murchie *et al.*, 2005; Rodríguez *et al.*, 2003; Yordanov and Angelova, 2010). HPP is carried out with intense pressure in the range of 100-1000 MPa, allowing most foods to be preserved with minimal effect on taste, texture, appearance, and nutritional characteristics (Patterson *et al.*, 2006; Torres and Velazquez, 2005; Yordanov and Angelova, 2010). This aspect is the main advantage of HPP compared to thermal sterilization and pasteurization because the HPP-treated food products maintain the sensory and nutritional characteristics (Yordanov and Angelova, 2010) (Lado and Yousef, 2002).

HPP has been used with success in the chemical, ceramic, and plastic industries for decades, but the food industry did not recognize its potential application until the middle of the 1980s (Table 6) (O'Reilly *et al.*, 2001; Otero and Sanz, 2003).

Table 6. Land marc events in the history of HPP for food products. Adapted from Patterson *et al*. (2006) and supplemented with information's from Food ingredients first (2011); Nguyen *et al*. (2010); Tonello (2011).

Year	Event(s)
1895	Royer (France) used high pressure to kill bacteria experimentally
1899	Hite (USA) used high pressure for food preservation
1980s	Japan started producing high-pressure jams and fruit products
1990s	Avomex (USA) began to produce high-pressure guacamole from avocados with a fresh taste and extended shelf-life
2000	Mainland Europe began producing and marketing fresh fruit juices (mainly citrus) and delicatessen-style cooked meats. High-pressure self-shucking oysters, poultry products, fruit juices and other products were marketed in the USA
2001	HPP fruit juices given approval for sale in the UK. Launch of the first HPP fruit

	juices in the UK
2003	Espuña (Spain) launched a line of ready-to-microwave HPP meat snacks (e.g. bacon and cheese rolls). In 2005, the company developed the first high pressure sliced cured ham
2008	Fonterra (New Zeeland) developed a pressurized antibody rich colostrum beverage
2009	FDA approval of the Pressure-Assisted Thermal Processing (PATP) for production of low acid foods, such as mashed potatoes
2011	Starbucks acquired Evolution Fresh with the aim of bringing premium HPP juices to the marketplace

HPP is gaining popularity with food processors not only because of its food preservation capacity but also because of its potential to achieve interesting function effects (Kadam *et al.*, 2012). Nevertheless, one century ago, Hite (1899) at West Virginia University Agricultural Experimental Station had already investigated the application of high pressure and published the first detailed report as a means of preserving milk. He reported that milk "kept sweet for longer" after a pressure treatment of ~650 MPa for 10 minutes at room temperature (Deliza *et al.*, 2005; Otero and Sanz, 2003; Patterson *et al.*, 2006). Hite *et al*. (1914) later reported that pressure could be used to extend the shelf-life of fruits, concluding that fruits and fruit juices responded well to high pressure because the "yeasts and other organisms having most to do with decomposition are very susceptible to pressure, while other organisms not so susceptible do not survive long the acid media" (Patterson *et al.*, 2006). Approximately eighty years later, Japan re-discovered the high pressure interest in this industry. From then, HPP has been adapted to the specific requirements of food industry (Otero and Sanz, 2003), gaining importance in this industry (Murchie *et al.*, 2005). Following initial successes with fruit juices and jams, the technology has been applied to an increasing range of food products, including smoothies, ham, guacamole, salsa, rice products, fish, and shellfish (Mújica-Paz *et al.*, 2011; Murchie *et al.*, 2005). Recently, a number of other HPP food products have been launched, including oysters in the USA, orange juice in France, and guacamole in Mexico (Huppertz *et al.*, 2002). HPP foods are produced and commercialized in several countries, and it is considered a technology with the most promising perspective of industrial utilization (Deliza *et al.*, 2005).

Over the past two decades, a lot of research in HPP applied in food has been developed (Figure 5), mainly dealing with pressure as a preservation method, to change the physical and functional properties of food systems (Otero and Sanz, 2003). One of the main advantages of this process is the almost instantaneous and isostatic pressure

transmission to the product, independent of size, shape, and food composition yielding highly homogenous products (Deliza *et al.*, 2005).

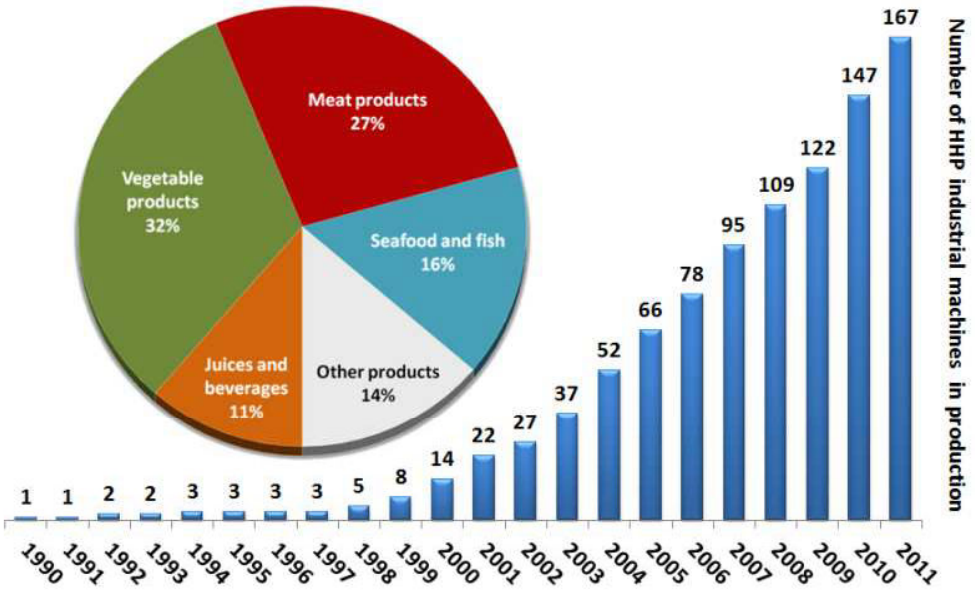

Figure 5. Data of world growth and main applications of HPP in the food industry. Courtesy of Hiperbaric, Burgos, Spain.

Recent studies reported that HPP has a strong positive influence on consumer interest, while, for comparative purposes, irradiation and genetic modification have extremely low interest among consumers (Wright *et al.*, 2007). Aspects such as nutritional quality, micro-biological safety, agrochemical residue, and environmental pollution are all examples of consumers concerns. Consumers nowadays are more interest than ever before in nutritious, healthy, and convenient foods which are possible by HPP (Deliza *et al.*, 2005).

Thus, HPP-treated products continue to proliferate in the global marketplace largely depending on the initiatives of food companies and the positive responses of consumers to these products. Commercial food applications of HPP have focused primarily on the ability of pressure to inactivated spoilage organisms and relevant foodborne pathogens and extend product shelf-life. While HPP successfully extends product shelf-life, consumer preferences ultimately determine the success of individual products in the marketplace. HPP has been used for a number of successful commercial

products, primarily because HPP treated foods, in addition to being microbiologically safe, retain more of their original fresh taste, texture, and nutritional content, such that these products are often superior in quality compared to their thermal processing counterparts. HPP can also confer unique benefits to products that impart them with advantages in the marketplace (Wright *et al.*, 2007).

In recent years, the use of HPP as a food preservation technique has gained momentum throughout the world as an alternative to traditional heat-based methods, for the reasons cited earlier (Fonberg-Broczek *et al.*, 2005; Hogan *et al.*, 2005), such as extend shelf-life, guarantee safety, and maintain fresh quality (Deliza *et al.*, 2005).

3.1.1. Principles and operation

HPP at refrigeration, ambient or moderate heating temperature allows inactivation of pathogenic and spoilage microorganisms in foods with fewer changes in texture, colour and flavour as compared to conventional technologies (Heinz and Buckow, 2010; Torres and Velazquez, 2005). The effect of HPP, unlike that of thermal processes and other conventional conservation technologies, is almost instantaneous and uniform throughout a system (McInerney *et al.*, 2007; Mújica-Paz *et al.*, 2011). Consequently, in contrast to thermal processing, products are treated evenly throughout, regardless of the packaging shape or volume of the product (Murchie *et al.*, 2005).

There are two general scientific principles of direct relevance to the use of HPP in food (Ramaswamy *et al.*, 1999). The first is Le Chatelier's Principle, which applies to all physical process and states that, when a system at equilibrium is disturbed the system responds in a way that tends to minimize the disturbance (Cheftel, 1995; Ledward, 1995). This means that HPP stimulates reactions that result in a decrease in volume but opposes reactions that involve an increase in volume. Any phenomenon (e.g. phase transition, change in molecular configuration, and chemical reaction) that is accompanied by a decrease in volume will be enhanced by pressure (Hogan *et al.*, 2005).

Secondly, the Isostatic Principle states that pressure is instantaneously and uniformly transmitted throughout a sample, whether the sample is in direct contact with the pressure medium or hermetically sealed in a flexible package that transmits pressure (Earnshaw, 1996). As can be seen in Figure 6, the pressure is transmitted in a uniform

(isostatic) and quasi-instantaneous manner throughout the sample. The time necessary for pressure processing is therefore independent of sample size and geometric because the pressure transmission to the core is not mass/time dependent, consequently the process is minimized in contrast to thermal processing (Hogan *et al.*, 2005; Yordanov and Angelova, 2010). With increasing pressure, the food reduces in overall size in proportion to the pressure applied but retains its original shape (Olsson, 1995; San Martin *et al.*, 2002). The application of pressure reduces microbiological load, including pathogens and spoilage organisms, leading to a high quality food with a significantly longer and safer chilled shelf-life (Patterson *et al.*, 2006; Shouqin *et al.*, 2004).

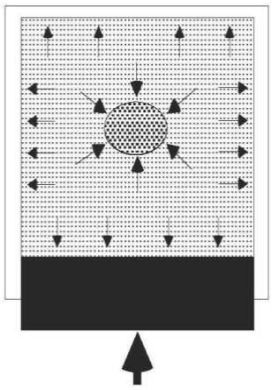

Figure 6. The principle of Isostatic processing (Yordanov and Angelova, 2010).

Chemical changes in HPP treated foods are minimal because the break of covalent bonds does not occur. Therefore, sensory properties, nutrients, and particularly bioactive compounds of current high commercial interest, suffer no significant losses. Pressure affects weaker bonds such as van der Waals forces, electrostatic interactions, and hydrogen bonds. Changes to them explain the preservation effect of HPP treatments (Huppertz *et al.*, 2002; Mújica-Paz *et al.*, 2011).

A HPP system consists of a high-pressure vessel and its closure, pressure-generation system, temperature-control device, and material-handling system (Huppertz *et al.*, 2002; Palou *et al.*, 2007; Yordanov and Angelova, 2010). The systems were initially developed in the chemical and material process industries for applications such as making artificial diamonds and sintered materials from powders. It is only during the past two decades that the food industry has begun using pressure treatment for food preservation. HPP is primarily practiced as a batch process where pre-packaged food

products are treated in a chamber surrounded by water or another pressure-transmitting fluid (most widely used fluids are glycol solutions, silicone oil, sodium benzoate solutions, ethanol solutions, inert gases, and castor oil). Semi-continuous systems have been developed for pumpable foods where the product is compressed without a container and subsequently packaged "clean" or aseptically (Hogan *et al.*, 2005; Palou *et al.*, 2007; Yordanov and Angelova, 2010).

The selection of equipment depends on the kind of food product to be processed. Solid food products or foods with large solid particles can only be treated in a batch mode. Liquids, slurries and other pumpable products have the additional option of semi-continuous production. Currently, most HPP machines in industrial use for food processing are batch systems, whereby the product is placed in a high-pressure chamber and the vessel is closed, filled with pressure chamber, and the vessel is closed. The vessel is filled with pressure-transmitting medium and pressurized either by pumping medium into the vessel or by reducing the volume of the pressure chamber, for example by using a piston (Hogan *et al.*, 2005). After the required holding time has elapsed, the system is depressurize, the vessel opened and the product unloaded. Then, the system is reloaded with product, either by operators or machines, depending on the degree of automation possible. The total time for pressurization, holding, and depressurization is referred as the "cycle time" (Hogan *et al.*, 2005). Pressurization and de-pressurization cycles can be rapid, allowing shorter processing times in comparison to thermal processing (Murchie *et al.*, 2005; Palou *et al.*, 2007). The cycle time and the loading factor (i.e. the percentage of the vessel volume actually used for holding packaged product, primarily a factor of package shape) determines the throughput of the system. In a commercial situation, with this sort of batch process, a short holding time under pressure is desirable in order to maximize throughput of product (Hogan *et al.*, 2005). For most applications, products are held for 3-5 min at 600 MPa. Approximately 5-6 cycles per hour are possible, allowing time for compression, de-compression, loading, and unloading (Yordanov and Angelova, 2010). For any HPP system, the working pressure is a very important parameter, not only because the initial price of the equipment increases significantly with its maximum working pressure, but also because a decrease in working pressure can reduce significantly the number of failures, increasing the working life of the equipment. Keeping the sample under pressure for extended periods of time does not require any additional energy. The work of

compression during HPP treatment will increase the temperature of foods through adiabatic heating, by approximately 3 °C per 100 MPa, depending on the composition of the food (Hogan *et al.*, 2005).

Batch processing reduces the risk of food large quantities becoming contaminated by either the lubricants or wear particles from the machinery. Different types of food can be processed in a batch system, without the danger of cross-contamination or the need to clean the equipment after each run. A technical advantage of the batch-type pressure vessel is the simplicity of fabrication when compared to a continuous flow pressure vessel operating at pressure as high as 400-900 MPa. A batch system pressure vessel with a processing capacity of 600 L/h of liquid food at a maximum operating pressure of 420 MPa was used to commercially produce grapefruit juice in Japan (Palou *et al.*, 2007).

3.1.2. Effects of high pressure processing on bovine milk

Thermal treatment of milk has been historically used in dairy industry and continues to be a very effective public safety measure. Depending on microbiological status, milk can be processed by various heating method resulting in pasteurized or sterilized products that are well defined by the regulators. Demands for longer shelf-life and wider distribution of milk and milk products have resulted in the concept of the Extended Shelf-Life milk (ESL) milk. However, there is no true definition of ESL milk and methods that can be used to produce it. Milk distribution in raw, refrigerated or shelf-stable categories differs greatly in various countries in developed, and less developed rural parts of the world. The interest in higher quality and extended shelf-life, potential energy savings resulted in broad development of alternative technological solutions for milk processing.

HPP treatment is currently of great interest in food research, primarily as an alternative to thermal processing for food products preservation (Huppertz, Smiddy*, et al.*, 2006). Although milk was the first food product to be treated with HPP (Hite, 1899) and considerable research attention has focused on HPP-induced changes in milk, however, HPP-treated milk is not commercially available thus far (Huppertz, Smiddy*, et al.*, 2006).

HPP is a promising method used to extend the shelf-life of milk and other food products. In addition to inhibition or destruction of microorganisms, HPP has an influence on the physical and chemical properties of milk (Permanyer *et al.*, 2010). It is known that milk treatments based on HPP between 300 and 600 MPa cause inactivation of microorganisms including most infectious foodborne pathogens without causing many modifications of endogenous milk enzymes and important quality characteristics such as taste, flavour, colour, vitamin, and nutrient content (Cheftel, 1995; Trujillo *et al.*, 2000; Trujillo *et al.*, 2002). Nevertheless, some milk constituents undergo physic-chemical modifications leading to changes in their functional properties (Iametti *et al.*, 1997; Law *et al.*, 1998).

The effects of heat treatment on milk proteins have been studied extensively over the last 50 years or so, but HPP induced changes in milk proteins has become of interest only over the last 15 years or so (Huppertz, Fox*, et al.*, 2006).

Overall, the structures of large molecules, such as proteins (including enzymes), may change under the influence of pressure. However, small molecules that have little secondary, tertiary and quaternary structure, such as amino acids, vitamins, and flavour/aroma components contributing to the sensory and nutritional quality of food, remain unaffected (Huppertz *et al.*, 2002).

Treatment of bovine milk at pressures over 100 MPa at 25 °C leads to a progressive β-Lg denaturation, being observed by the loss of solubility at pH 4,6, while α-La and BSA are resistant to pressures up to 400 MPa (Lopez-Fandino *et al.*, 1996). With treatments at 200 and 400 MPa, at room temperature for 15-30 min, β-Lg was denatured (14-16% and 82-90%, respectively). A pressure of 600 MPa for 15-30 min denatured 15-33% of α-La. The extent of denaturation is dependent on the pressure level, holding time and temperature. The protein α-La was more resistant to denaturation under pressure than β-Lg (López-Fandiño, 2006).

III. Objectives

The aim of this work was to study the effect of thermal and HPP pasteurization, and subsequent refrigerated storage (4 °C) on donkey milk and colostrum on:

- Indigenous microflora: total microflora, *Enterobacteriaceae* and coliforms;
- Antimicrobial enzyme activity: lysozyme;
- Protein composition: immunoglobulin A, M and G.

This objective was defined as to be the first study on the possibility of donkey milk pasteurization for human and infant consumption, as an alternative to human milk or infant formulas, by the application of high pressure processing technology.

IV. Materials and Methods

1. Milk collection and preparation

Donkey milk and colostrum samples were obtained from Quintinha do Silval in Vila Nova de Anços (Coimbra), in February 2013 and were kindly supplied by his proprietary (Pedro Gonçalves). The milk samples were collected from three healthy donkeys and colostrum milk was collected from one healthy donkey. Then, the milk samples were mixed and kept at 4 °C. The colostrum sample was also kept at 4 ºC.

In sterile conditions, the milk and colostrum samples were divided in different aliquots to evaluate the thermal and HPP pasteurization. A control sample of raw untreated milk was also used. After processing, half of the samples (milk and colostrum) were stored at -80 °C to evaluate the lysozyme activity and immunoglobulin content and the other half of the samples were stored at 4 °C to study the microbiological load during 30 and 40 days for milk and colostrum, respectively.

2. Thermal pasteurization

The samples were thermal pasteurized by the Holder or LTLT (Low Temperature Long Time) pasteurization method (62.5 °C for 30 min), according to the general HMBANA procedure (Updegrove, 2005). Samples of donkey milk (5.0 mL) and colostrum (3.0 mL) were added in polypropylene tubes and placed in a thermostatic bath at 62.5 °C. The time needed for the milk reach 62.5 °C was previously estimated in bovine milk, using a thermocouple to measure the temperature in the center of the sample. Bovine milk was used instead of donkey milk and colostrum to avoid spending of the samples. Once 62.5 °C temperature was reached, the samples were held in the thermostatic bath for 30 min. Following pasteurization, the tubes were quickly cooled in ice slurry.

3. High pressure processing treatments

HPP treatments were carried out using a hydrostatic press (High pressure system, Model U33, Unipress Equipment, Poland). This equipment has a pressure vessel of 100 mL (35 mm diameter and 100 mm height) surrounded by an external

jacket, connected to a thermostatic bath (Huber Compatible Control CC1, New Jersey, USA) to control the temperature and a mixture of propylene glycol and water (60:40) was used as pressurizing fluid. The unit has a maximum working pressure of 700 MPa and a working temperature between -20 and 100 °C.

Nine different treatments were performed by combining three pressure levels with three holding times: 400 MPa, 550 MPa, and 625 MPa, for 2.5, 10 and 30 min each. The initial temperature of the pressure vessel was set to 8 °C. Samples of donkey milk (5 mL) and colostrum (3 mL) were aseptically packed in low permeability polyamide-polyethylene bags (PA/PE-90, Albipack-Packaging Solutions, Águeda, Portugal), vacuum-sealed to keep out air, and inserted into an additional individual bag made of same material, with caution not to leave air inside the two bags. After the HPP-treatment, all group samples were transferred to cold water for rapid cooling.

4. Microbiological analysis

The microbiological quality of donkey milk and colostrum samples was assessed, using traditional microbiological methods and media for the enumeration of Aerobic Mesophilic Bacteria, *Enterobacteriaceae* and Total Coliforms (Tasci, 2011).

4.1. Samples preparation and dilution

Of each sample, 1.0 mL was obtained aseptically and homogenized with 9.0 mL sterile Ringer's solution. Further, decimal dilutions were made with the same diluent and duplicates of dilutions were plated on the appropriate media, according to the following procedures.

4.2. Aerobic mesophilic bacteria counts

Total aerobic mesophilic counts at 30 °C were determined in plate count agar (PCA, Merck), following the standard method NP 4405 (*Microbiologia Alimentar. Regras gerais para contagem de microrganismos 30°C, in NP 4405, IPQ, Editor 2002: Lisboa.*) / ISO 4833:2003 (*Standard, N., ISO 4833: 2003 Microbiology - General*

Guidance for the enumeration of microorganisms. Colony count technique at 30°C.), being the pour-plated method used with 1.0 mL of diluted solution sample. The plates were incubated at 30 ± 1 °C for 72 ± 3 h and the yellow colonies formed were counted.

4.3. *Enterobacteriaceae* counts

Enterobacteriaceae counts were quantified in violet red bile dextrose agar (VRBDA, Merck), by the pour plated method, being incubated aerobically at 37 ± 1 °C for 24 h, and counted the red-pink colonies formed, according to the standard method NP 4137:1991/ ISO 21528:2004 (*Microbiologia Alimentar. Regras gerais para determinação de Enterobactereaceae sem revitalização. Técnicas do número mais provável (NMP) e de contagem de colónias, in NP 4137:1991 / ISO 21528:2004, IPQ, Editor: Lisboa.*) (Saletti *et al.*, 2012).

4.4. Total coliforms counts

Total Coliforms and *E. coli* counts were enumerated in Chromocult® Coliform Agar (CCA, Merck), by the pour-plated method, being incubated aerobically at 37 ± 1 °C for 24 h. Pink colonies were classified as Total Coliforms, whereas dark blue colonies were classified as presumptive *E. coli* colonies, according to the standard method NP 4137:1991/ ISO 21528:2004 (AOAC, 2002; *Microbiologia Alimentar. Regras gerais para contagem de microrganismos 30°C, in NP 4405, IPQ, Editor 2002: Lisboa.*).

4.5. Microbial counts

Petri dishes containing 30-300 colony forming units (CFU) were selected for counting, according to ISO 4833:2003. The microbial counts were calculated following the equation (1):

$$N = \frac{\sum Characteristic\ colonies}{V[(n_1 + 0.1 \times n_2) \times d]} \qquad \textbf{(Equation 1)}$$

being:

N – Colony forming units per mL of sample (CFU / mL)

V – Sample volume (mL)

n_1 – Number of plates in the 1st dilution

n_2 – Number of plates in the 2nd dilution

d – 1st dilution

The results were converted into logarithmic decimals of the number of CFU per mL of sample. All samples were analysed in duplicate.

5. Immunoglobulin content analysis

Immunoglobulins A, M and G were measured in donkey milk and colostrum using Human IgA, IgM and IgG ELISA kits (KOMA BIOTECH, Seoul, Korea), according to the manufacturer's instructions. ELISA (or Enzyme-Linked Immunosorbent Assay) is an immunoassay technique involving the reaction of antigen and antibody *in vitro*. These kits are based on the sandwich ELISA type, elucidated in Figure 7. In order to describe the general procedures of the kits, the letter X will be used as a generic designation for any of the three Igs. The kits contained all of the required reagents and material for IgX quantitation: a pre-coated 96 well ELISA microplate (with antigen-affinity purified goat anti-human IgX), plate sealers, detection antibody (horseradish peroxidase conjugated antigen-affinity purified goat anti-human IgX), standard protein (human reference serum), assay diluent (1% bovine serum albumin), colour development reagents (tetramethylbenzidine and H_2O_2 solutions), stop solution (2 M H_2SO_4), and washing solution (phosphate buffered saline powder with 0.05% Tween-20, pH 7.4).

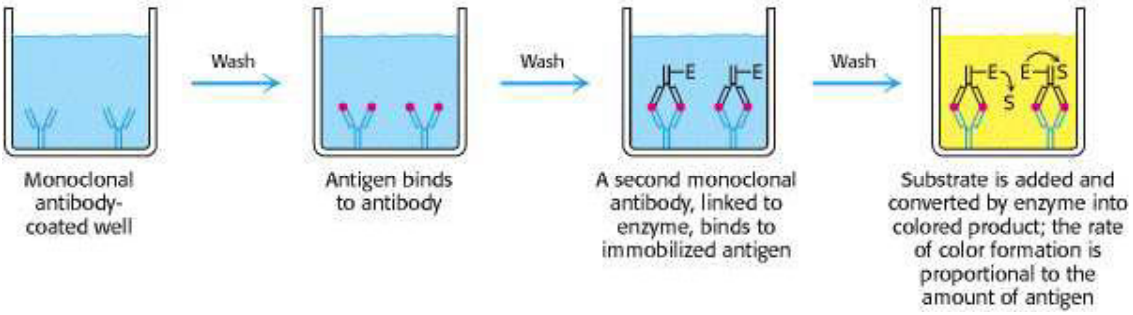

Figure 7. General steps of the sandwich ELISA assay.

In all of the procedures, 100 µL of blank, standard or donkey milk or colostrum samples were added to each well of the pre-coated microplate and incubated at room temperature for 1 h with the plate sealer provided. The standards were diluted following the manufacturer's recommended dilutions. Then, 100 µL of the diluted detection antibody (1:20000 to 1:50000) were added per well and incubated during 1 h at room temperature in the sealed plate. Before, between and after the above steps the wells were aspirated to remove the liquid and washed five times with the washing solution to remove unbound molecules. For the colour development reaction, 100 µL of colour development solution were added to the wells and incubated for about 10 min. The reaction was stopped by adding 100 µL of the stop solution and the absorbance read at 450 nm in less than 20 min using a Multiskan GO microplate Spectrophotometer (Thermo Scientific, Waltham, USA). Duplicate determinations were performed for each Ig, and total concentrations were determined using the standard curves constructed with the diluted standards (Appendix A).

6. Lysozyme activity analysis

Lysozyme activity was determined using *Micrococcus lysodeikticus* based turbidimetric procedure recommended by Sigma Chemical Co. (Sigma, Missouri, USA), with minor modifications. The principle of this assay is the lytic activity of lysozyme towards *Micrococcus lysodeikticus* cell walls (antibacterial activity), by measuring the loss of light intensity in the direction of incident beam propagation, with reference to a standard solution. A 0.015% (w/v) *Micrococcus lysodeikticus* cell suspension (substrate) was prepared by suspending *Micrococcus lysodeikticus* ATCC 4698 lyophilized cells in sodium phosphate buffer (66 mM, pH 6.24). To assure the

freshness of this suspension, its absorbance at 450 nm (A_{450nm}) was measured at the beginning of the analyses and was consistently between 0.6 and 0.7, as it should. Prior to the enzymatic reaction, the substrate was heated at 30 °C in a thermostatic bath. The reaction was initiated by adding 0.10 mL of appropriately diluted donkey milk or colostrum to 2.50 mL of substrate and, after mixing by inversion, the decrease in A_{450nm} was immediately recorded for 3 min at 10 s intervals using a PerkinElmer Lambda 35 UV-Vis spectrophotometer (PerkinElmer Instruments, Massachusetts, USA). Measurements were carried out against the reagent blank, containing the substrate and 0.10 mL of buffer, and each sample was measured in triplicate. The ΔA_{450nm}/min was obtained using the initial linear rate. One unit of lysozyme activity was defined as the amount of the enzyme that produces a ΔA_{450nm}/min of 0.001 per min at pH 6.24 at 30 °C using a suspension of *Micrococcus lysodeikticus* as substrate, in a 2.6 mL reaction mixture.

7. Statistical analysis

Differences between treatments were tested at a 0.05 level of significance. The effects of pressure level and holding time were tested in a one-way analysis of variance (ANOVA), followed by a multiple comparisons test (Tukey's HSD) to find which treatments were significantly different from one another. All data are expressed as the "mean ± standard deviation". The standard deviation was always ≤ 10%.

8. Kinetic data analysis

Denaturation kinetics of milk and colostrum Igs toward HPP was subjected to reaction kinetic analysis. Isobaric denaturation of donkey milk IgG at 400 and 550 MPa could be described by a first order model **(2)**. Donkey colostrum IgG at 550 and 625 MPa, and IgM at 550 MPa could also be described by a first order model **(2)**. According to equation **(2)**, loss of Ig concentration rate *(-dA/dt)* is proportional to the denaturation rate constant (*k*) and the Ig concentration at each treatment time (*A*).

$$\frac{dA}{dt} = -kA \quad \textbf{(2)}$$

The reaction rate constant was determined from a semilogarithmic plot **(3)** of the Ig retention (A/A_0) as a function of the exposure time (t). A represents the response value after HPP treatment and A_0 is the initial value. D-values (decimal reduction time) were also calculated according to **(4)**.

$$\ln\left(\frac{A}{A_0}\right) = -kt \quad \textbf{(3)}$$

$$D = \frac{\ln(10)}{k} \quad \textbf{(4)}$$

V. Results and Discussion

Part I: Microbiological analysis

1.1. Microbial quantification in donkey colostrum and milk

The microbiological load of milk has a decisive effect on the quality and safety of dairy products (Szteyn *et al.*, 2005). Milk contaminated by high levels of spoilage bacteria usually becomes unsuitable for further processing since it does not meet the consumer's expectations in terms of health (nutritional value), safety (hygienic quality) and satisfaction (sensory attributes) (Mhone *et al.*, 2011).

1.1.1. Aerobic mesophilic bacteria counts

- Colostrum:

Analyzing Figure 8, the initial total aerobic mesophiles in raw donkey colostrum (day 0) was below the detection limit (<1.0 log CFU/mL), increasing to 6.73 log CFU/mL at day 4. After 21 days of storage, the total aerobic mesophiles ranged was 8.03 log CFU/mL, showing the microbiological growth in the raw donkey colostrum samples. After the day 21 of storage no more microbiological quantifications of total aerobic mesophiles, were performed since it was observed that the samples of raw donkey colostrum showed a very high microbiological load.

In the treated samples (thermal and HPP pasteurization), it was verified that the total microbial counts remained unchanged during 40 days of storage, having been obtained values below the detection limit (< 1.0 log CFU/mL).

The results obtained are according to other authors (Tonello *et al.*, 1992), who observed that HPP induced microbial inactivation (3.0 log CFU/mL) in raw bovine colostrum. These authors treated raw bovine colostrum at 200 MPa and 20 °C for 16 hours, and in this study the samples were treated at 400 and 550 MPa and 4 °C for 10 min.

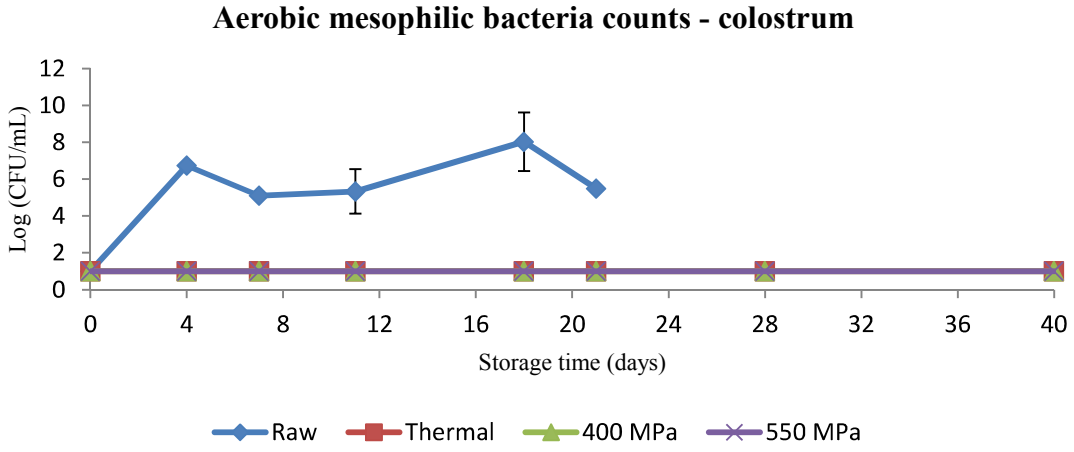

Figure 8. Total aerobic mesophilic microorganisms after 0, 4, 7, 11, 18, 21, 28 and 40 days of storage at 4 °C in raw donkey colostrum and after thermal and HPP pasteurization of donkey colostrum.

- Milk:

Concerning donkey milk, the total microbial counts in raw samples remained unchanged during 3 days of storage (< 1.0 log CFU/mL), showing an increase to 5.31 ± 0.23 log CFU/mL at 6 days and to 7.94 ± 0.16 log CFU/mL at 9 days, remaining unchanged until the day 23. These results were similar to those obtained by Šarić *et al.* (Šarić *et al.*, 2012) that observed a microbiological count of 6.41± 0.21 log CFU/mL after 6 days of storage. According to a report by other authors (Zhang *et al.*, 2008), raw donkey milk contained a low total microbial counts with an average of 4.34 ± 0.37 log CFU/mL.

The results obtained for the samples treated by thermal pasteurization (Figure 9) showed an increase to 2.28, 2.37 and 4.30 log CFU/mL at days 13, 20 and 23 days, respectively. The results obtained at day 0 were similar to those obtained in another work (Chiavari *et al.*, 2005) (<1.0 log CFU/mL). In the samples treated with HPP the microbiological load was always below the detective limit (<1.0 log CFU/mL) during 30 days of storage.

Permanyer *et al.* (2010) verified the degree of microbial inactivation in human milk achieved by HPP. HPP treatments (400, 500 and 600 MPa for 5 min at 12 °C) reduced the total bacterial counts to undetectable levels, regardless of the initial

microbial load and the pressure level. These authors obtained excellent results and confirmed the efficacy of HPP for microorganisms inactivation.

In conclusion, it was possible to verify the same profile in both samples (donkey colostrum and milk): the total aerobic mesophilic bacteria in untreated samples increased during the storage time to unacceptable limits, whereas in HPP treated samples the initial microbiological counts (<1.0 log CFU/mL) remained unchanged, with better results being observed, compared to the thermal treatment (in the case of milk).

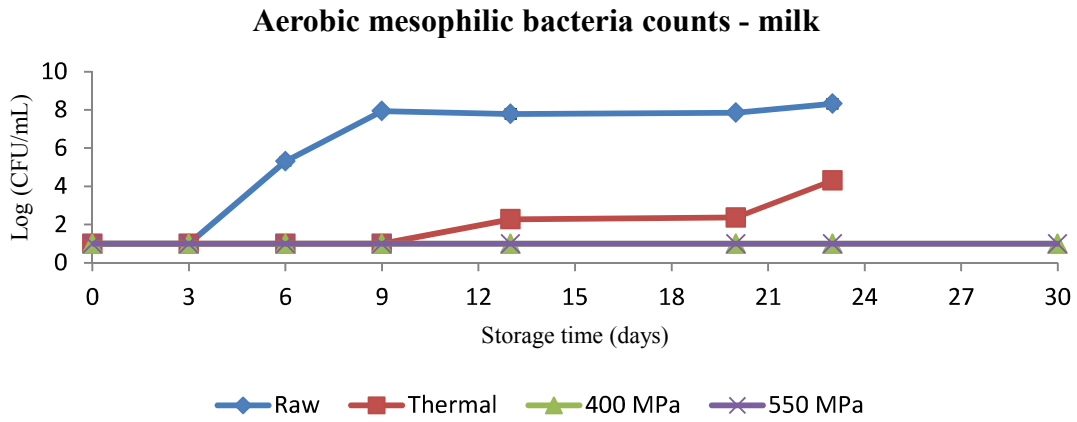

Figure 9. Total aerobic mesophilic microorganisms after 0, 3, 6, 9, 13, 20, 23 and 30 days of storage at 4 °C in raw donkey milk and after thermal and HPP pasteurization of donkey milk.

1.1.2. *Enterobacteriaceae* counts

- Colostrum:

According to Figure 10, *Enterobacteriaceae* in raw donkey colostrum were not detected until day 4 (<1.0 log CFU/mL). However, after 7 days there was a gradual increase to 3.48, 4.35 and 8.18 log CFU/mL at 7, 11 and 18 days, respectively. In both treated samples (thermal and HPP pasteurization), it was verified that the *Enterobacteriaceae* counts remained unchanged during 40 days of storage and below the detection limit (<1.0 log CFU/mL).

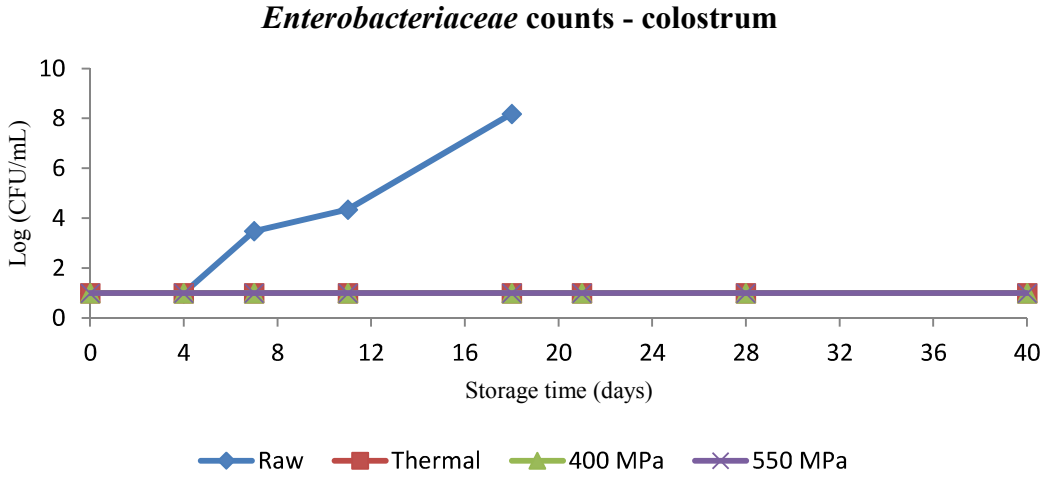

Figure 10. *Enterobacteriaceae* counts in raw donkey colostrum and treated samples (thermal and HPP pasteurization) during 40 days storage at 4 °C.

- Milk:

Relatively to raw milk, *Enterobacteriaceae* counts remained unchanged during 3 days of storage (<1.0 log CFU/mL), rising to 3.35, 5.00, 5.00, 7.20 and 7.02 log CFU/mL at 6, 9, 13, 20 and 23 days, respectively (Figure 11).

A similar behaviour was already reported in the literature by Šarić *et al*. (2012), who observed an increase of the *Enterobacteriaceae* counts in raw donkey milk during storage, of 0.60 to 1.04 log CFU/mL at 3 to 6 days, respectively. As can be seen in Figure 10, the results obtained in raw donkey milk were higher to those reported in the literature, for *Enterobacteriaceae* counts. At day 0, Coppola *et al.* (2002) reported 3.2 log CFU/mL, while Chiavari *et al*. (2005) reported 2.01 log CFU/mL.

It was possible to verify that with HPP the *Enterobacteriaceae* counts (Figure 11) in donkey milk, remained unchanged during the 40 days of storage and below the detection limit (<1.0 log CFU/mL). This result is not in accordance with those of Huppertz, Smiddy*, et al.* (2006), who quantified 3.0 log CFU/mL in bovine milk treated at 400 MPa for 30 min and 25 °C. The milk samples treated, in this study, with thermal pasteurization showed an increase after 23 days of storage to 4.04 log CFU/mL.

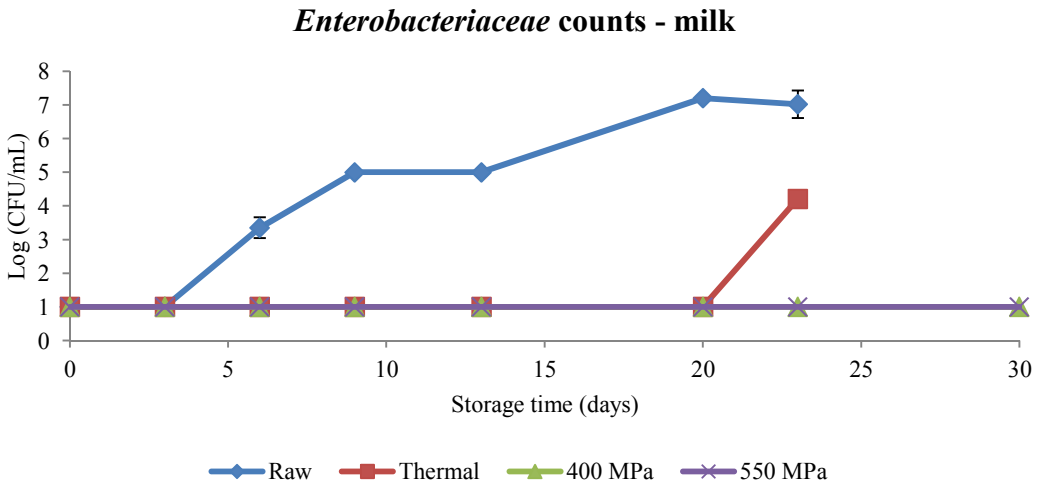

Figure 11. *Enterobacteriaceae* counts in raw donkey milk and treated samples (thermal pasteurization, 400 and 550 MPa for 10 min at 8 °C) during 30 days storage at 4 °C.

1.1.3. Total coliforms counts

The presence of total coliforms in foods of animal origin indicates environmental sources of contamination since these microorganisms are abundant in the environment (Szteyn *et al.*, 2005). Amongst the coliforms, *Escherichia coli* is the most common contaminant of raw and processed milk (Mhone *et al.*, 2011). It is a reliable indicator of fecal contamination of water and food such as milk and dairy products.

- Colostrum:

In raw donkey colostrum, the coliforms counts were below the detection limit in all cases during all the storage period (40 days) and for both treatments (thermal and HPP pasteurization), and for the raw colostrum.

- Milk:

In the case of raw donkey milk (Figure 12), coliforms counts were below the detection limit until day 23 (<1.0 log CFU/mL), but, after this day there was an increase to 3.00 log CFU/mL. Coppola *et al.* (2002) reported an initial coliforms count in donkey milk of 1.4 log CFU/mL. Zhang *et al.* (2008) reported a higher count of coliforms after

4 days of storage (3.7 log CFU/mL), and Šarić *et al.* (2012), verified an increase of 0.85 and 1.00 log CFU/mL after 5 and 6 days of storage, respectively. In the present work, the samples treated by thermal pasteurization, showed an increase after 23 days to 4.04 log CFU/mL.

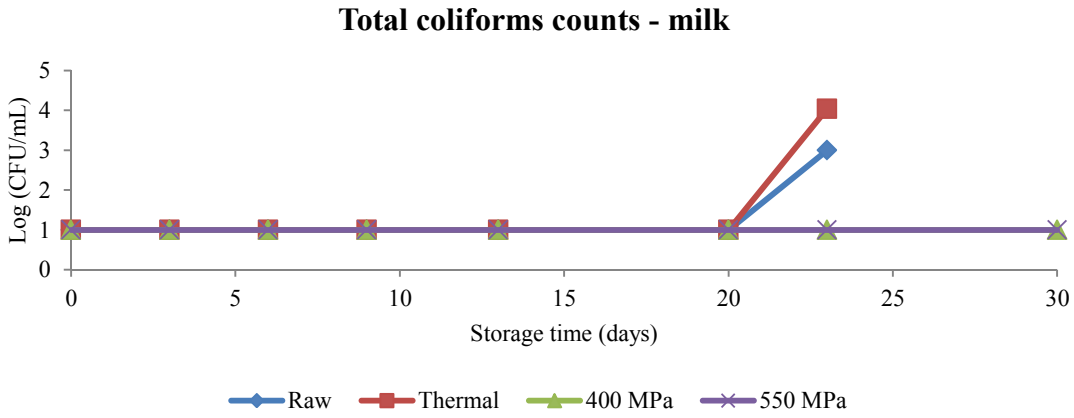

Figure 12. Coliforms counts in raw donkey milk and treated samples (thermal pasteurization, 400 and 550 MPa for 10 min at 8 °C) during 30 days storage at 4 °C.

In conclusion, the results obtained indicated that HPP can be a potential alternative for the pasteurization of donkey colostrum and milk.

Part II: Antimicrobial enzyme activity

2.1. Effects of thermal pasteurization and HPP on lysozyme activity of donkey colostrum

Lysozyme is one the most important natural antimicrobial enzyme in milk (Shahani *et al.*, 1980), and lysozyme is present in donkey milk in high quantities (Vincenzetti *et al.*, 2008). This enzyme, together with other factors including Igs, lactoferrin, and lactoperoxidase, may function in the infant's digestive tract to reduce the incidence of gastrointestinal infections (Businco *et al.*, 2000) and may contribute to the inhibition of bacterial growth (Cosentino *et al.*, 2013; Salimei *et al.*, 2004). Significant losses in lysozyme content after thermal pasteurization have been reported for human colostrum (≈ 74%) by Koenig *et al.* (2005a), and, the results obtained by these authors were similar to those of Velona *et al.* (1999), who observed a decreased of 65 to 85%. Viazis *et al.* (2007) reported a 21% loss of lysozyme activity in mature human milk after thermal pasteurization.

Lysozyme activity in raw and treated samples (by thermal and HPP pasteurization) of donkey colostrum is presented in Figure 13. The percentage and content of lysozyme activity retention found in this study for donkey colostrum after thermal pasteurization and pressure treatments is presented in Appendix C.

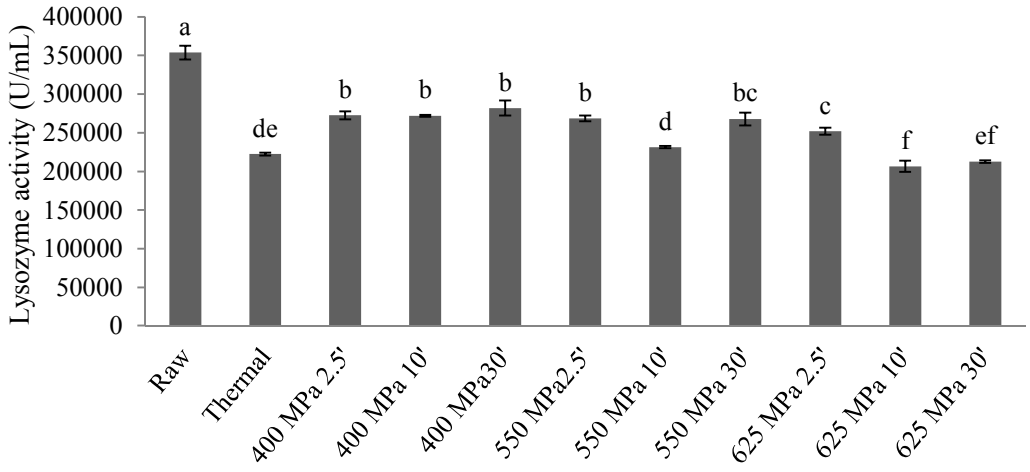

Figure 13. Concentration of lysozyme activity in donkey colostrum before and after thermal pasteurization and HPP treatments.

The initial lysozyme activity in raw colostrum was higher than the activity observed in donkey milk (353700 U/mL *vs*. 53240 U/mL, respectively). Similar conclusions were found by Qureshi and Enbergs (2012) that showed that the colostral lysozyme activity was higher than that observed in milk (203278 U/mL *vs*. 34340 U/mL, respectively).

The initial lysozyme activity decreased significantly to 63% ($p<0.05$) after thermal pasteurization of donkey colostrum. A similar conclusion was found by Koenig *et al*. (2005), who verified that lysozyme content in human colostrum after thermal pasteurization decreased to ≈74%.

HPP treatment caused in all cases a significant reduction ($p<0.05$) in lysozyme activity compared to the raw unprocessed colostrum (Figure 12). Nonetheless, the percentage of lysozyme activity retention ranged between 77 and 80% after pressure treatments of 400/550 MPa (2.5, 10 and 30 min) and 625 MPa (2.5 min), respectively, being the decrease lower than the observed after thermal pasteurization (63%). Treatments of 625 MPa for 10 and 30 min caused a decrease ($p<0.05$) of lysozyme activity (58 and 60%, respectively), similar to values observed for thermal pasteurization.

No data are available on the literature on lysozyme activity in donkey colostrum to discuss these results. As compared to human beings and other animals (like cow, sheep, and goat), the average lysozyme activity in donkey colostrum is higher (Nikkhah, 2012; Zhang *et al.*, 2008). These results are very interesting, since lysozyme content in donkey colostrum may contribute to safety of donkey colostrum for increased periods.

2.2. Effects of thermal pasteurization and HPP on lysozyme activity of donkey milk

Donkey milk has noteworthy antimicrobial characteristics due to the high concentration of lysozyme and other natural inhibitory substances, like lactoferrin than confer high hygienic qualities (Tidona *et al.*, 2011).

Lysozyme activity in raw and treated samples of donkey milk is presented in Figure 14. The content and percentage of lysozyme activity retention found in this study for donkey milk after thermal pasteurization and pressure treatments are presented in Appendix D.

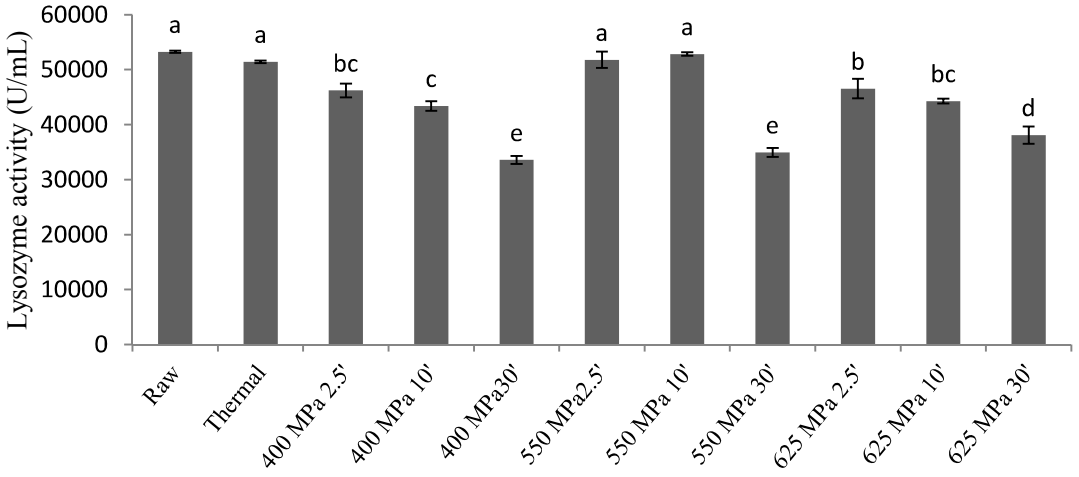

Figure 14. Concentration of lysozyme activity in donkey milk before and after thermal pasteurization and the various HPP treatments studied.

Thermal pasteurization did not affected ($p>0.05$) the lysozyme activity in donkey milk. In human milk different results were reported. Evans *et al.* (1978) demonstrated that lysozyme activity decrease after thermal pasteurization by 24%, while Czank *et al.* (2009) found a 61% destruction of lysozyme in mature human milk. Viazis *et al.* (2007) reported a 21% loss of activity after thermal pasteurization, whereas Uniacke-Lowe *et al.* (2010) verified that equine milk lysozyme is more stable to denaturation than human lysozyme after thermal pasteurization at 62 °C for 30 min, but at 71 °C for 2 min or 82 °C for 15 s, inactivation was similar in both.

Comparatively to other animals, lysozyme in cow's milk has been extensively studied. There is a common agreement that cow's milk contains a very low concentration of lysozyme (0-58.5 U/mL). This has been justified by the fact that lysozyme is released by broken neutrophils in serum and cow's neutrophils contain extremely low concentration of lysozyme (Qureshi and Enbergs, 2012).

Relatively to HPP effect on donkey milk, only the pressure treatments at 550 MPa for 2.5 and 10 min did not cause significant differences ($p>0.05$) in lysozyme activity compared to the raw unprocessed milk samples (Figure 13). On the other hand, the lysozyme activity retention found in pressure treatments at 400 MPa for 2.5 and 10 min, and 625 MPa for 2.5 and 10 min ranged between 81% and 87%. HPP treatments of 400 and 550 MPa for 30 min caused a decrease to 63% and 66%, respectively. No data on literature is available on lysozyme activity in donkey's milk to discuss these results.

Presently, the only enzyme that has been analyzed after HPP of human milk is lysozyme (Viazis *et al.*, 2007), but studies of the effect of HPP on bovine milk reveal that the most indigenous milk enzymes are quite baroresistant. As reviewed by Huppertz, Fox*, et al.* (2006), most milk enzymes like plasmin, alkaline phosphatase, lactoperoxidase, xanthine oxidase, phosphoisomerase, ɤ-glutamyltransferase and lipase are resistant to pressures up to 400 MPa.

2.2.1. HPP kinetics denaturation of donkey milk on lysozyme activity

As decreases in lysozyme activity were detected in donkey milk after HPP treatment at 400 and 550 MPa, these data were subjected to reaction kinetic analysis and could be described by a first order kinetic model (Appendix F). The rate of lysozyme activity denaturation increased with pressure and treatment time. The denaturation kinetic parameters *k* (reaction rate constant, min^{-1}) and D (decimal reduction time, min) were calculated for HPP at 400 and 550 MPa and are presented in Table 7 (Ludikhuyze *et al.*, 2001; Tayefi-Nasrabadi *et al.*, 2011).

Table 7. Kinetic parameters for HPP denaturation of lysozyme in donkey milk.

	Pressure (MPa)	*k*-value (min^{-1})	r^2	D-value (min)
Lysozyme	400	1.19×10^{-2}	0.994	193.49
	550	1.56×10^{-2}	0.907	147.60

D-value at 550 MPa was lower than that at 400 MPa (1.3-fold). These results give valuable preliminary information and should be considered in future studies about the lysozyme activity/content in donkey milk.

Part III: Immunoglobulins content analysis

Igs form an important component of the immunological activity found in milk and colostrum. The content of Igs in colostrum and milk is highly dependent on the animal species (Butler and Kehrli, 2005; W. L. Hurley, 2003b). Analyzing Figure 2, IgG is the principal Ig in equine and bovine colostrum and milk, while IgA is the principal form in equine milk and human colostrum and milk (Uniacke-Lowe *et al.*, 2010).

3.1. Effects of thermal pasteurization and HPP on immunoglobulins of donkey colostrum

Many milk processing protocols include thermal treatment of the colostrum, milk or whey. Chen and Chang (1998) research in bovine milk indicated that Igs are thermolabile (Zagorska and Ciprovica, 2012). Exposure to high temperatures can reduce detectable bovine IgG by 40% (75 °C for 5 min), and 100% (95 °C for 15 s) (Chen and Chang, 1998). Mainer (1997) reported that IgG was the most thermostable and IgM the least thermostable in bovine milk (Mainer *et al.*, 1997).

Table 8. Total IgA, IgM and IgG concentrations in raw colostrum samples.

Protein	Raw colostrum samples ± SD
Immunoglobulin G (mg/mL)	$1.67 \times 10^{-6} \pm 8.21 \times 10^{-8}$
Immunoglobulin A (mg/mL)	$2.03 \times 10^{-6} \pm 2.81 \times 10^{-7}$
Immunoglobulin M (mg/mL)	$2.28 \times 10^{-5} \pm 6.61 \times 10^{-7}$

In this study, Igs concentrations obtained for the untreated raw colostrum (Table 8) showed different values compared to mare colostrum. W. L. Hurley (2003a) found a high IgG concentration of 113.4 mg/mL, a IgA concentration of 10.7 mg/mL and a lower IgM concentration of 5.4 mg/mL, and the differences compared to the values of the present work can be due to the different species studied. There are no data reported

in the literature on the content of Igs on donkey colostrum and milk respectively, only in equine that includes mare and donkeys.

Others authors analyzed human milk and obtained different results. Ronayne de Ferrer *et al.* (1984) found a higher IgA concentration of 2.128 mg/mL in human milk after 1-10 days postpartum (colostrum and transitional milk), but even higher IgA concentrations in colostrum (≈ 8-9 mg/mL) were found by others authors (Ramírez-Santana *et al.*, 2012; Yuen *et al.*, 2012). IgM concentration found by Shi *et al.* (2011) was 0.12 mg/mL, lower than that reported by Mickleson and Moriarty (1982) with an average of 0.58 mg/mL on day 3. The IgG content obtained by Mickleson and Moriarty (1982) was of 0.19 mg/mL on day 3, but Shi *et al.* (2011) reported a higher IgG concentration of 0.46 mg/mL.

Some studies have already investigated the concentration of Igs on equine colostrum and milk, but there are no studies that investigated the effect of thermal pasteurization and HPP effects on donkey milk and colostrum, and this is the first one to assess high pressure and thermal pasteurization. Actually, the thermal pasteurization technique is the most usually employed pasteurization procedure in different type of milks and colostrum. Figure 14 shows the IgG, IgM and IgA concentration in donkey colostrum before and after thermal pasteurization and for a wide range of pressures and holding times. IgG, IgM and IgA concentrations (mg/mL) can be also seen in Appendix B, in a table format to depict the concentration values and the errors associated.

The pressure treatments used by Sousa *et al.* (2014) for human colostrum show that IgA content is retained after HPP at 200 and 400 MPa for 2.5, 10 and 30 min, and 600 MPa for 2.5 min at 8 °C. Increasing the hold time at 600 MPa for 15 and 30 min resulted in significant IgA losses of 20 and 26%, respectively. These authors found no significant reduction of IgM and IgG in colostrum for a wide range of pressures and holding times (200 and 400 MPa for all studied holding times, and 600 MPa for 2.5 min for IgM), but the pressure treatments at 600 MPa for 15 and 30 min caused a significant destruction of IgM (59 and 60%, respectively) and IgG (35 and 40%, respectively). The results obtained by Sousa *et al.* (2014) confirms that HPP between 200 and 400 MPa up to 30 min maintains not only IgA content in human colostrum, but also IgM and IgG concentrations.

Analyzing Figure 15, most of the pressure treatments and thermal pasteurization studied caused variations in Igs concentration on donkey colostrum. In the case of IgG,

pressure treatments at 550 MPa for 2.5 and 10 min, and 625 MPa for 2.5 and 10 min caused a significant increase ($p<0.05$) of 2, 1.4, 4.1 and 2.6-fold, respectively.

Regarding the highest pressure treatment (625 MPa for 30 min) the IgG content was significantly reduced ($p<0.05$) by 47%. After the thermal treatment a decrease (79%) of IgG concentration was observed ($p<0.05$) (Figure 15).

IgM concentration decreased ($p<0.05$) after thermal and HPP pasteurization. HPP caused the highest decrease of concentration compared to thermal treatment, being observed values below detection limit at 625 MPa for 10 and 30 min.

All treatments applied (thermal pasteurization and HPP) caused a reduction of IgA to below the quantification limits.

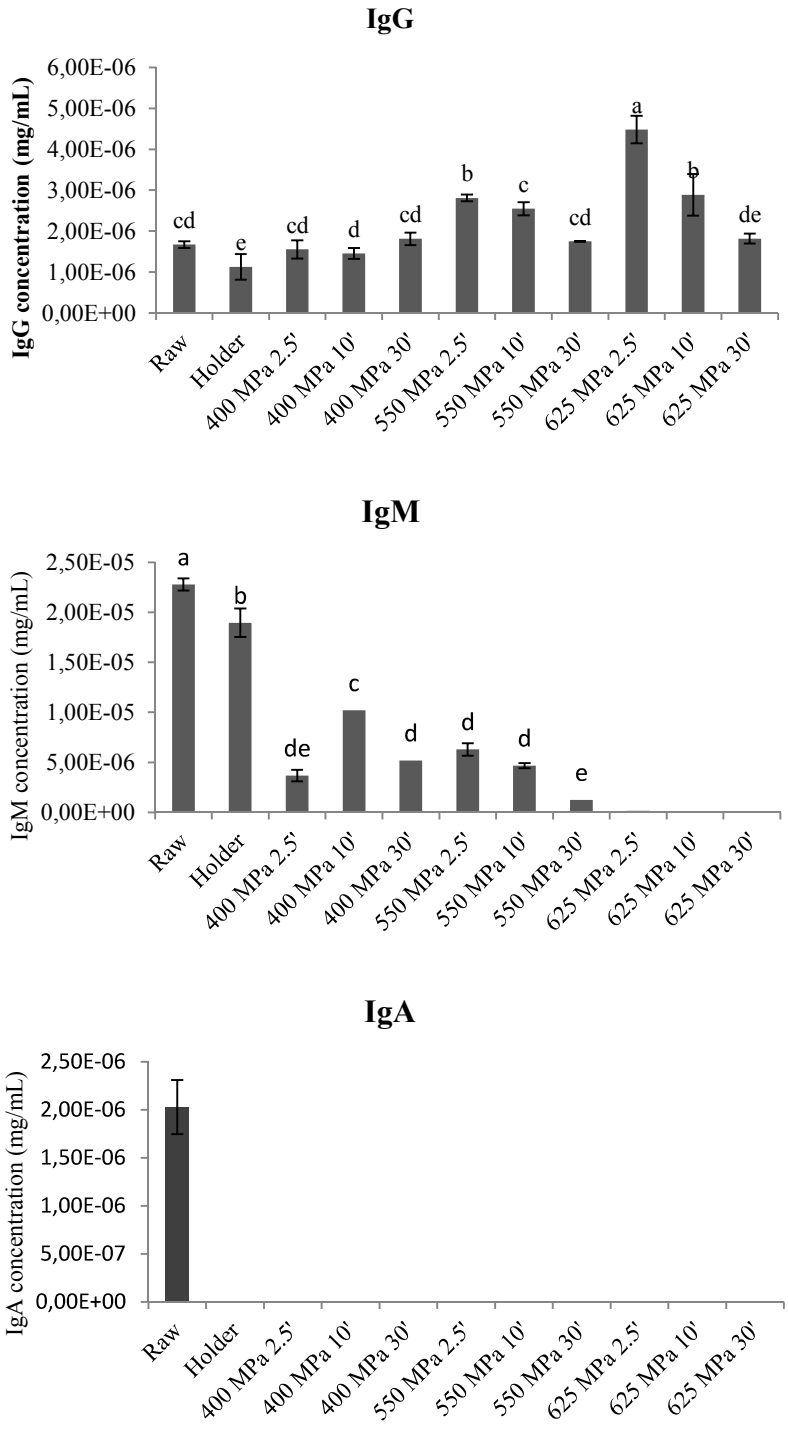

Figure 15. Concentration of IgG, IgM and IgA in donkey colostrum before and after thermal pasteurization and HPP treatments.

To our knowledge there are no reported data of HPP and thermal pasteurization effects on Igs concentration of donkey colostrum. The Ig content in human colostrum after HPP and thermal treatment was affected differently depending on the treatments applied. Diverse authors reported variable losses of these immune factor after thermal pasteurization, i.e., Ford *et al.* (1977) found an IgA reduction of 20%, and, more recently, Permanyer *et al.* (2010) and Franch *et al.* (2010) obtained a similar decrease of 28% in the IgA content. Very high reductions in the concentration of Igs were also observed by Koenig *et al.* (2005b) in thermal pasteurized colostrum (the losses were 61% for IgA, IgG and 72% to 100% for IgM). While Evans *et al.* (1978) obtained 34% reduction of IgG content after pasteurization of mature milk, Koenig *et al.* (2005a) found more than twice as much IgG destruction (≈ 72%) in colostrum.

In contrast, in this study the thermal pasteurization significantly reduced IgG and IgM initial concentration to 21 and 83%, respectively. It was verified that IgM is the most pressure labile Ig (lower D-value) and IgG the most pressure stable (higher D-value).

3.1.1. HPP kinetics denaturation of donkey colostrum immunoglobulins

Slight decreases in Igs concentration were detected after HPP of donkey colostrum at 550 MP and significant losses were found after 625 MPa with increasing treatment time. These data were subjected to reaction kinetic analysis and could be described by a first order kinetic (Appendix D). Data of IgG concentration at 400 MPa and for IgM at 400 and 625 MPa could not be adjusted to this model, as no linear decrease of Igs retention (A/A_0) with increasing treatment times was observed. The denaturation kinetic parameters k (reaction rate constant, min^{-1}) and D (decimal reduction time, min) were calculated for HPP at 550 and 625 MPa and are presented in Table 9.

Table 9. Kinetic parameters for HPP denaturation of IgG and IgM in donkey colostrum, assuming a first order kinetics.

Immunoglobulin	Pressure (MPa)	*k*-value (min^{-1})	r^2	D-value (min)
IgG	550	2.31×10^{-2}	0.910	99.68
	625	7.57×10^{-2}	0.997	30.42
IgM	550	6.05×10^{-2}	0.992	38.06

The D-value of IgG for 550 MPa was 2.6-fold higher than IgM, which means that treatments at 550 MPa require more than double the time to reduce the IgG concentration in one logarithmic cycle than IgM. For IgG the difference between the pressure treatments is even higher, D-value at 550 MPa is about 3.3-fold higher than 625 MPa. The results obtained for values of D-values at 550 MPa confirm that IgG is more stable than IgM.

To our knowledge, there are no reported data of high pressure effects on Igs in donkey colostrum. However, a study of thermal denaturation of buffalo milk Igs reported IgA as being the most thermal labile Ig, followed by IgM (EL-Loly *et al.*, 2007).

3.2. Effects of thermal pasteurization and HPP on immunoglobulins in donkey milk

HPP effects on donkey milk IgG, IgA and IgM contents for a wide range of pressures and holding times were determined. Thermal pasteurization was also tested with the purpose of comparing the effects of both technologies. Table 10 presents IgG, IgA and IgM concentrations in raw donkey milk.

Table 10. Total IgA, IgM and IgG concentrations in raw milk samples.

Protein	Raw milk samples ± SD
Immunoglobulin G (mg/mL)	$1.15 \times 10^{-6} \pm 9.15 \times 10^{-8}$
Immunoglobulin A (mg/mL)	$9.92 \times 10^{-7} \pm 2.31 \times 10^{-8}$
Immunoglobulin M (mg/mL)	$4.18 \times 10^{-6} \pm 1.28 \times 10^{-6}$

In this work, Igs concentrations obtained for the untreated raw milk (Table 9) are different than those of other authors on mare milk. W. L. Hurley (2003a) found a high IgG concentration of 0.39 mg/mL, a IgA concentration of 0.48 mg/mL and a lower IgM concentration of 0.03 mg/mL, but this author found these results in mare milk.

Figure 16 shows the IgG, IgM and IgA concentration in donkey milk after thermal pasteurization and for a wide range of pressures and holding times. IgG, IgM and IgA concentrations (mg/mL) before and after all of the treatments can be seen in Appendix B.

According to Figure 16, the IgG concentration was increase 1.5-fold ($p<0.05$) after HPP treatment at 400 MPa at 2.5 min. However, HPP treatments of 400 MPa for 30 min, 550 and 625 MPa caused a decrease ($p<0.05$) of IgG concentration between 50% to below detection limit.

Regarding thermal pasteurization, it caused a significant decrease ($p<0.05$) to 56% in donkey milk IgG content.

Relatively to IgM and IgA, HPP treatments caused a decrease ($p<0.05$) of Igs concentrations to below the detection limit, except at 400 MPa for 2.5 min and 625 MPa for 10 min, being observed a IgM retention of 15% in both treatments; and at 550 MPa for 10 min, being detected a IgA retention of 37%. In the case of thermal pasteurization, it is observed a decrease to 18 and 14% for IgM and IgA, respectively.

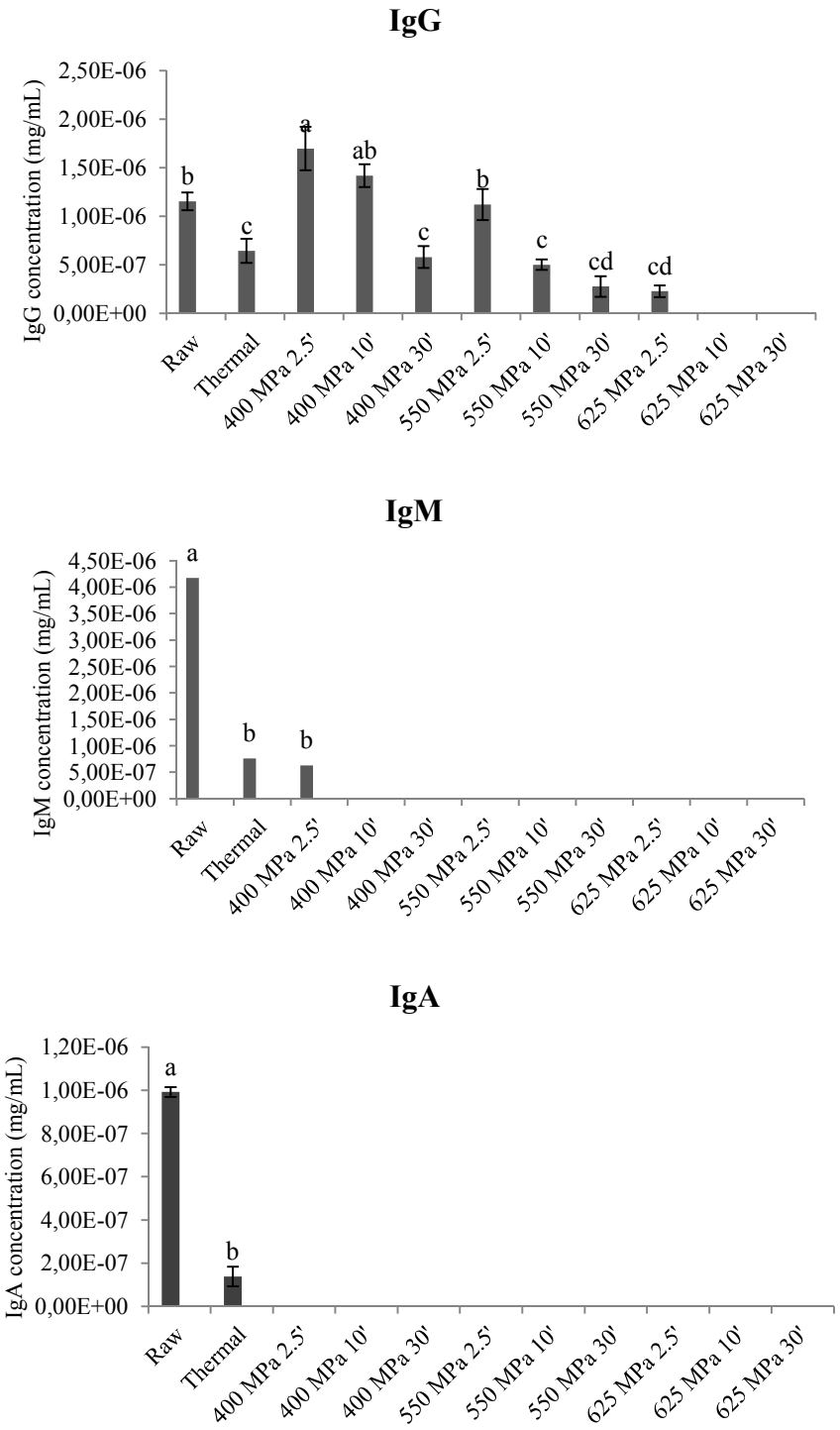

Figure 16. Concentration of IgG, IgM and IgA in donkey milk before and after thermal pasteurization and the HPP treatments performed.

To our knowledge there are no reported data of HPP and thermal pasteurization effects on Igs concentration on donkey milk. More recently, Contador *et al.* (2013) compared the effect of thermal pasteurization (62.5 °C for 30 min) with HPP treatments (at 400 and 600 MPa for 3 and 6 min) on the Igs (IgA, IgG and IgM) on human milk. These authors concluded for IgM and IgA, treatments at 400 MPa (3 or 6 min) maintained the initial levels in human milk, while treatment at higher pressure (600 MPa) or thermal treatment produced higher reductions (only 40-50% of the initial contents were preserved). With respect to IgG levels, the thermal pasteurization showed lower IgG content than that processed by HPP, although these differences were not significant ($p>0.05$). Overall, these authors concluded that HPP could be a suitable alternative for the preservation of Igs in human milk.

The effect of HPP on the IgM, IgA and IgG content of human milk was evaluated by Sousa *et al.* (2014). IgA content was retained after HPP at 200 and 400 MPa for 2.5, 15 and 30 min, and 600 MPa for 2.5 min. The same treatments were also effective in maintaining IgG concentration and IgG was not significantly reduced after any of the treatments at 200 and 400 MPa, and 600 MPa for 2.5 min. Relatively to IgM content, the same authors reported that HPP at 200 and 400 MPa for 2.5, 10 and 30 min did not cause significant reductions in IgM content ($p>0.05$). Nevertheless, after 600 MPa for 2.5 min, IgM content were significant reduced to 63%. After holder pasteurization, all Igs concentration was significantly reduced, mainly the IgM content, with a reduction in 69%.

However, other studies have evaluated the effect of HPP on the IgG content of caprine or bovine milk. In caprine milk, treatments at 400 or 500 MPa for 10 min (at 20 °C), reduced the IgG content by 20 and 38%, respectively (Trujillo *et al.*, 2007). Felipe *et al.* (1997) showed that Igs in caprine milk were stable at pressures of 300 MPa at 25 °C but were partially destroyed (35%) after HPP at 500 MPa. Li *et al.* (2006) studied bovine milk treated by HPP, and found that the IgG content was maintained at pressures lower than 276 MPa.

3.2.1. HPP kinetics of donkey milk immunoglobulins

As indicated above only HPP denaturation of IgG could be described by a first order kinetic model (Appendix E), after reaction kinetic analysis for all Igs. For IgG,

linear decreases were found only for the pressure treatments at 400 and 550 MPa. The denaturation kinetic parameters *k* (reaction rate constant, min^{-1}) and D (decimal reduction time, min) were calculated and are expressed in Table 11.

Table 11. Kinetic parameters for HPP denaturation of IgG in donkey milk, assuming a first order kinetic.

Immunoglobulin	Pressure (MPa)	*k*-value (min^{-1})	r^2	D-value (min)
IgG	400	4.02×10^{-2}	0.988	57.28
	550	4.66×10^{-2}	0.887	49.41

D-value at 400 MPa is about 1.2-fold higher than 550 MPa, which means that treatment at 400 MPa require more time to reduce the IgG concentration in one logarithmic cycle than treatments at 550 MPa.

VI. Conclusions

Cow's milk is a high nutritious food, especially for infants. However, cow's milk proteins are also a major food allergen, and tend to induce allergic reactions in infants. Prevention of milk allergy is an urgent problem all over the world and needs to be resolved through combined efforts of nutritionists, food scientists and physicians. For this reason, particular attention has been given to the donkey milk, because, in comparison with cow's milk, it has been less studied and can be considered the closest natural milk to human milk in composition.

In general, it was concluded that, when comparing thermal pasteurization with HPP, this method showed generally better results. The microbial analysis of colostrum and milk samples pasteurized showed an increase of all microbial groups. While in the colostrum and milk samples tested with HPP treatments this did not occur. Lysozyme activity revealed small changes between pressure processed and raw donkey colostrum and milk, unlike the results demonstrated with thermal pasteurization (decrease of lysozyme activity). Finally, when comparing HPP with thermal pasteurization for Igs retention, HPP at 400 and 550 MPa resulted in much higher retentions, while thermal pasteurization always caused significant decreases. These results show the great potential of HPP to be used in donkey milk production with long shelf-life, with better microbial and antimicrobial characteristics.

VII. Future work

Considering all the results obtained in the present work, other studies should be conducted in order to determine HPP effects in other nutritional and non-nutritional donkey milk components. It is essential to study HPP effects in donkey milk lipids, as well as fatty acid profile; whey proteins and caseins; carbohydrates in general (mainly lactose) and oligosaccharides in particular, as they have important bioactive functions. Effects on vitamins also have to be determined.

Further work should also focus on investigating HPP effects on other bioactive and immune factors, such as lactoferrin, lactoperoxidase, lipases (BSSL and LPL), amylase and growth factors.

However, more studies need to be carried out to understand in depth the effect of HPP on pathogenic microorganisms in donkey milk, and studied the effect of HPP on lactic acid bacteria, and yeast and moulds.

To complete the present research, it would be of major interest further clinical challenges by using donkey milk in children affected by CMPA, in order to better evaluate the effects of donkey milk in children with this pathology, and to compare in these patients the effects of donkey's milk with the effects of dairy cow's milk or infant formulae (normally based on dairy cow's milk).

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Appendices

Appendix A. Standard curves constructed for the determination of immunoglobulins in donkey colostrum and mature milk.

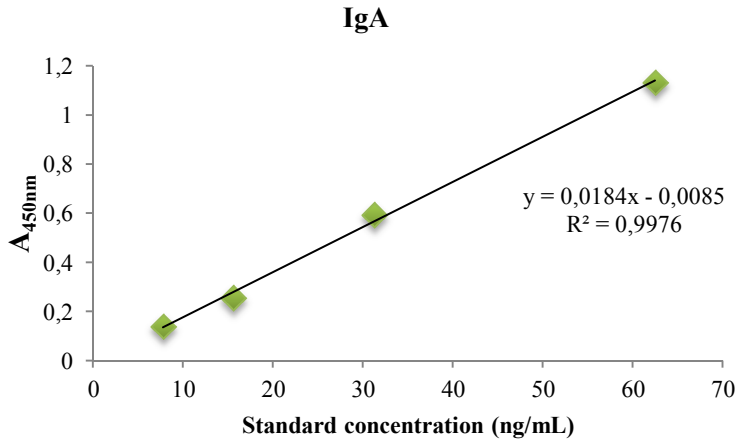

Figure A1. Standard curve used for determining IgA concentration in donkey colostrum and milk.

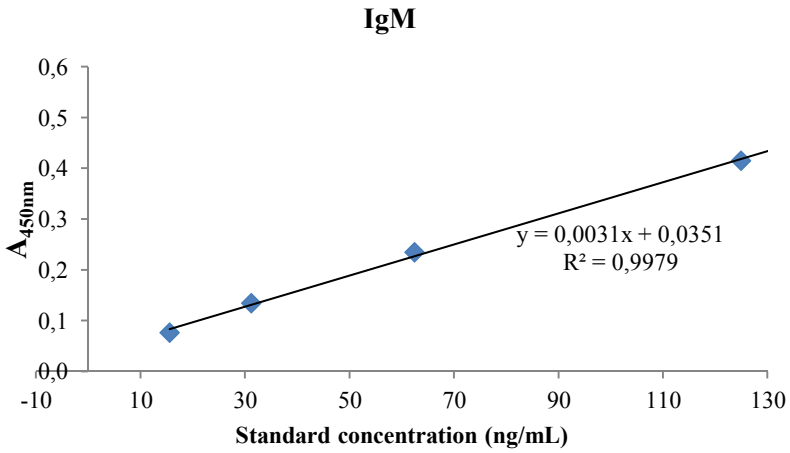

Figure A2. Standard curve used for determining IgM concentration in donkey colostrum and milk.

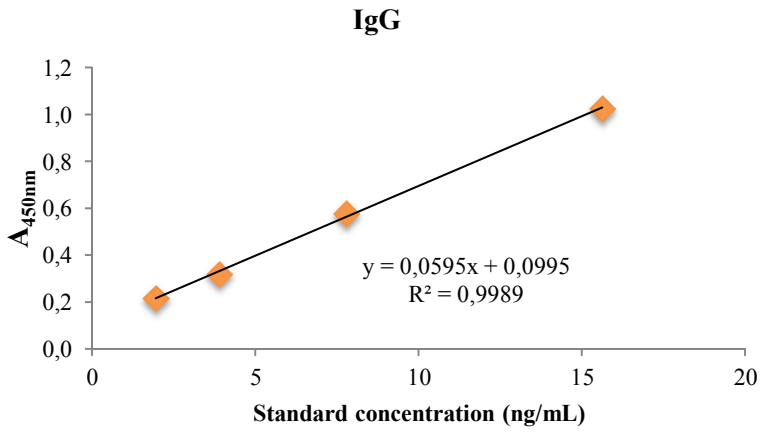

Figure A3. Standard curve used for determining IgG concentration in donkey colostrum and milk.

Appendix B. Immunoglobulin concentration in raw and treated colostrum and milk samples.

Table B1. Donkey colostrum IgA, IgM and IgG concentrations before and after Holder pasteurization and the various HPP treatments.

Treatment	IgA concentration (mg/mL)	IgM concentration (mg/mL)	IgG concentration (mg/mL)
Raw	2.03E-06 ± 2.81E-07	2.28E-05 ± 6.16E-07	1.67E-06 ± 8.21E-08
Holder	n. d.	1.90E-05 ± 1.41E-06	3.55E-07 ± 3.10E-07
400 MPa 2.5'	n. d.	3.68E-06 ± 5.70E-07	1.55E-06 ± 2.24E-07
400 MPa 10'	n. d.	1.02E-05 ± 4.79E-07	1.45E-06 ± 1.35E-07
400 MPa 30'	n. d.	5.18E-06 ± 1.92E-06	1.81E-06 ± 1.52E-07
550 MPa 2.5'	n. d.	6.29E-06 ± 6.16E-07	3.47E-06 ± 8.02E-08
550 MPa 10'	n. d.	4.66E-06 ± 3.65E-07	2.39E-06 ± 1.62E-07
550 MPa 30'	n. d.	1.24E-06 ± 1.82E-07	1.75E-06 ± 4.21E-09
625 MPa 2.5'	n. d.	1.61E-07 ± 1.60E-07	6.88E-06 ± 3.40E-07
625 MPa 10'	n. d.	n. d.	4.34E-06 ± 5.07E-07
625 MPa 30'	n. d.	n. d.	8.83E-07 ± 1.21E-07

*"n.d." – not detected

Table B2. Donkey milk IgA, IgM and IgG concentrations before and after Holder pasteurization and the various HPP treatments.

Treatment	IgA concentration (mg/mL)	IgM concentration (mg/mL)	IgG concentration (mg/mL)
Raw	9.92E-07 ± 2.31E-08	4.18E-06 ± 1.28E-06	1.15E-06 ± 9.15E-08
Holder	1.8E-07 ± 4.53E-08	7.60E-07 ± 0.00E+00	6.45E-07 ± 1.24E-07
400 MPa 2.5'	n. d.	6.30E-07 ± 0.00E+00	1.70E-06 ± 2.23E-07
400 MPa 10'	n. d.	n. d.	1.42E-06 ± 1.18E-07
400 MPa 30'	n. d.	n. d.	5.80E-07 ± 1.13E-07
550 MPa 2.5'	n. d.	n. d.	1.12E-06 ± 1.60E-07
550 MPa 10'	n. d.	n. d.	5.02E-07 ± 5.47E-08
550 MPa 30'	n. d.	n. d.	2.76E-07 ± 1.07E-07
625 MPa 2.5'	n. d.	n. d.	2.28E-07 ± 5.94E-08
625 MPa 10'	n. d.	n. d.	n. d.
625 MPa 30'	n. d.	n. d.	n. d.

*"n.d." – not detected

Appendix C. Lysozyme activity in raw and treated colostrum and milk samples.

Table C1. Lysozyme activity in raw and treated colostrum samples.

Treatment	Lysozyme Activity (U/mL)	Lysozyme Activity (%)
Raw	353700 ± 8743	100
Holder	222525 ± 1806	63
400 MPa 2.5'	272625 ± 5221	77
400 MPa 10'	271875 ± 1098	77
400 MPa 30'	281925 ± 9772	80
550 MPa 2.5'	268650 ± 3637	76
550 MPa 10'	231450 ± 1330	65
550 MPa 30'	267825 ± 8312	76
625 MPa 2.5'	251925 ± 4592	71
625 MPa 10'	206625 ± 7362	58
625 MPa 30'	212550 ± 1741	60

Table C2. Lysozyme activity in raw and treated milk samples.

Treatment	Lysozyme Activity (U/mL)	Lysozyme Activity (%)
Raw	53240 ± 183	100
Holder	51413 ± 231	97
400 MPa 2.5'	46207 ± 1232	87
400 MPa 10'	43387 ± 855	81
400 MPa 30'	33573 ± 743	63
550 MPa 2.5'	51787 ± 1489	97
550 MPa 10'	52820 ± 314	99
550 MPa 30'	34940 ± 819	66
625 MPa 2.5'	46547 ± 1780	87
625 MPa 10'	44280 ± 420	83
625 MPa 30'	38080 ± 1577	72

Appendix D. First order kinetic denaturation of donkey colostrum immunoglobulins.

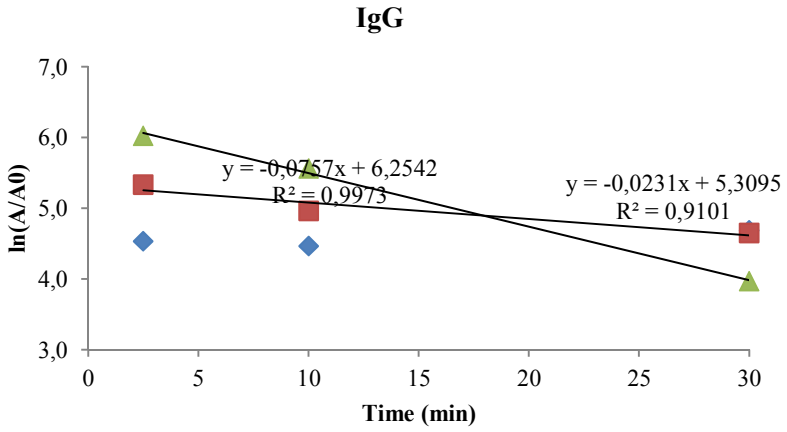

Figure D1. Effect of pressure treatment on donkey colostrum IgG concentration (A/A_0) as a function of treatment time at different pressures: 400 MPa (◆), 550 MPa (■) and 625 MPa (▲).

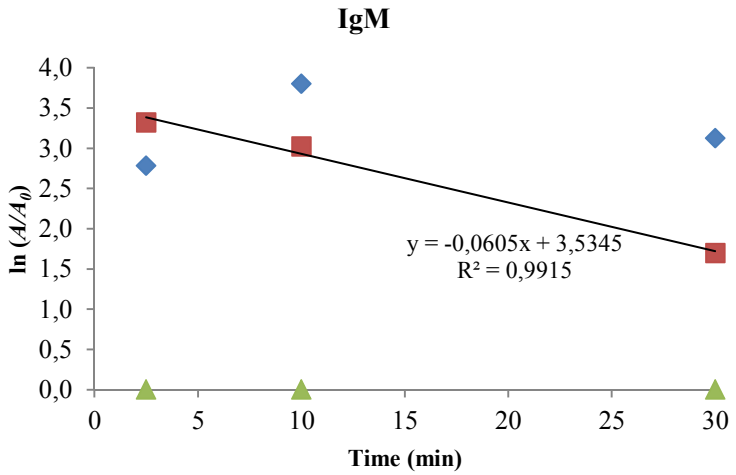

Figure D2. Effect of pressure treatment on donkey colostrum IgM concentration (A/A_0) as a function of treatment time at different pressures: 400 MPa (◆), 550 MPa (■) and 625 MPa (▲).

Appendix E. First order kinetic denaturation of donkey milk immunoglobulins.

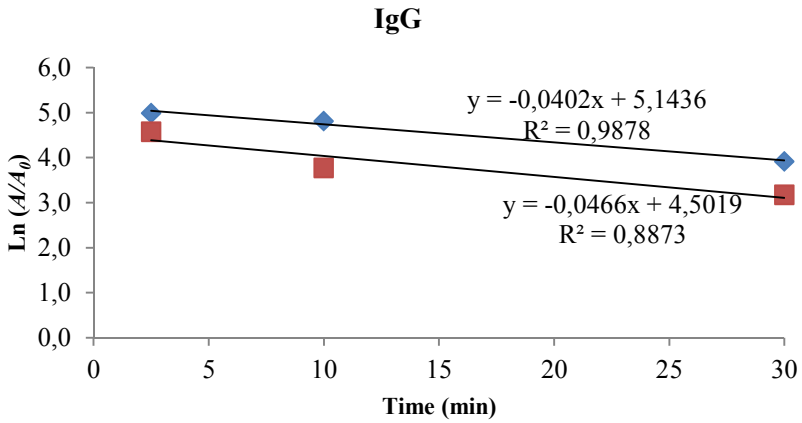

Figure E1. Effect of pressure treatment on donkey milk IgM concentration (A/A_0) as a function of treatment time at different pressures: 400 MPa ( ) and 550 MPa ( ).

Appendix F. First order kinetic denaturation of lysozyme activity in donkey milk.

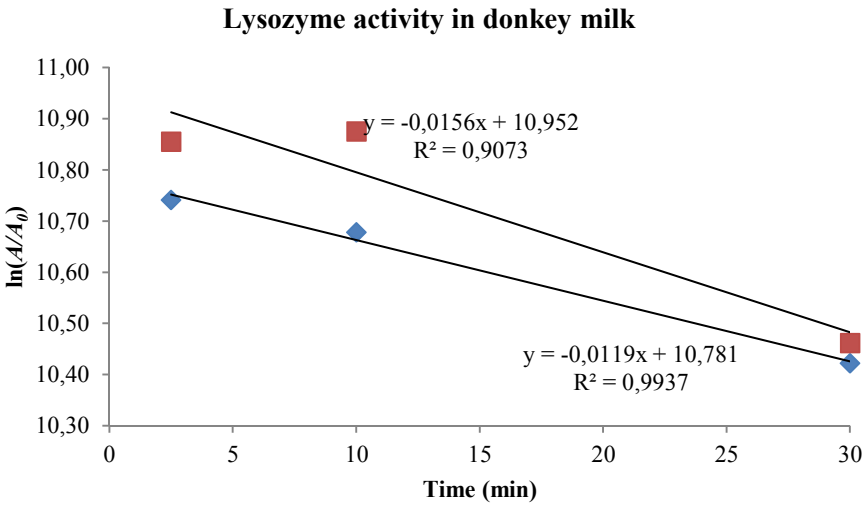

Figure F1. Effect of pressure treatment on donkey milk IgM concentration (A/A_0) as a function of treatment time at different pressures: 400 MPa ( ) and 550 MPa ( ).